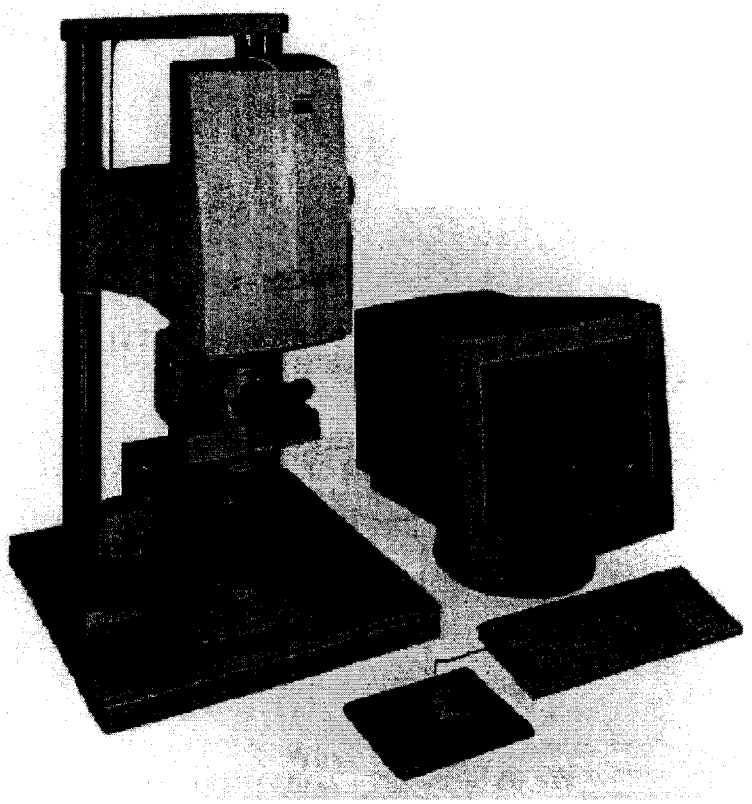
LaserSharp2000 for the MRC1024

Software Reference Manual



BIO-RAD

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1. PREFACE AND WARNINGS

Bio-Rad Microscopy Division
September 2000

LaserSharp2000 (for MRC-1024) Software Reference Manual, Issue 1.0

1.1 LaserSharp2000 Software - limited use licence conditions

The software described in this manual is supplied under a limited use licence agreement.

1.2 Software copyright

The LaserSharp2000 software is the sole and exclusive property of Bio-Rad. The customer undertakes not to copy any part of the software without written permission from Bio-Rad except for a back-up copy for security purposes.

1.3 Software use

The software may only be used on the machine for which it was originally supplied.

1.4 Unauthorized use of software

The customer agrees to protect the software from unauthorized use.

1.5 Software support limitations

The LaserSharp2000 software will only be supported when run on a Bio-Rad recommended computer (at the time of purchase).

1.6 Other products referred to in this manual

Windows NT and Windows 2000 are registered trademarks of Microsoft Corporation.

The Trademarks of all fluorochromes and probes are recognised.

1.7 Manual part number

Further copies of this manual may be obtained by quoting Manual Part Number 9MRC50UM05.

1.8 Symbols and conventions

<Enter> indicates the identification of a specific key on the computer keyboard.

File|Open... indicates a menu title on the menu bar followed by a menu item choice within that menu.

Drop-down menus: When you click on a menu title a drop-down menu appears.

Dropdowns: Click on the down arrow to select an option from a dropdown menu. This can also be an option in a drop-down menu, which produces a second menu.

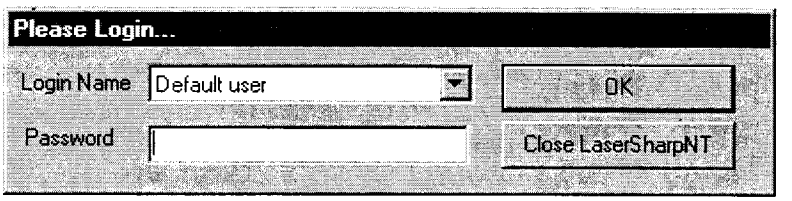
Pop-up menus: These context sensitive menus offer functionality relevant to the mouse position. They are accessed by right-clicking.

Spin box : Click on the left or right arrow to move the slider left or right. Alternatively, you can enter a value directly in the box provided alongside the slider or grab the slider with the mouse.

1.9 Starting the LaserSharp2000 software

To start the software double click the LaserSharp2000 icon:

The software will initially prompt you to Login. The system will be supplied with the Default user's password set to '1'.



Once you have successfully logged in the system will proceed with an initialisation phase during which firmware is 'downloaded' to microprocessors distributed around the system and system communication protocols are checked.

2. AN INTRODUCTION TO THE SOFTWARE

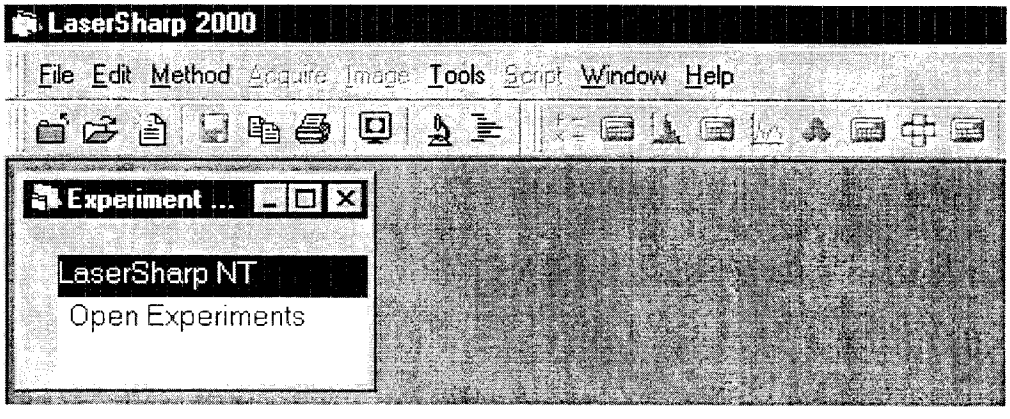
The LaserSharp software is run under Windows NT4.0™ operating system. Unlike the OS/2 version it is a unified application which controls acquisition and provides functionality for processing and analysis of images and data. The following text should be read by ***all*** users before attempting to use the system.

2.1 User Interface

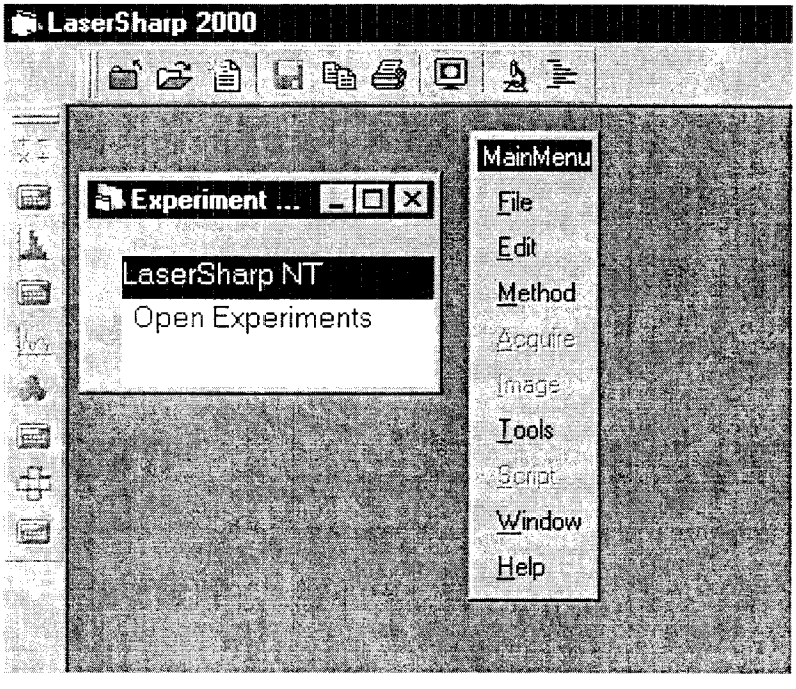
The user interface has been designed to be used with a minimum screen resolution of 1280 x 1024 pixels. It should be noted that under Windows NT it is possible to run this application with duall monitors (provided that the appropriate graphics card is available).

2.1.1 MAIN MENU AND TOOL BARS

The main menu bar and tool bars will initially appear in the top left hand corner as below:

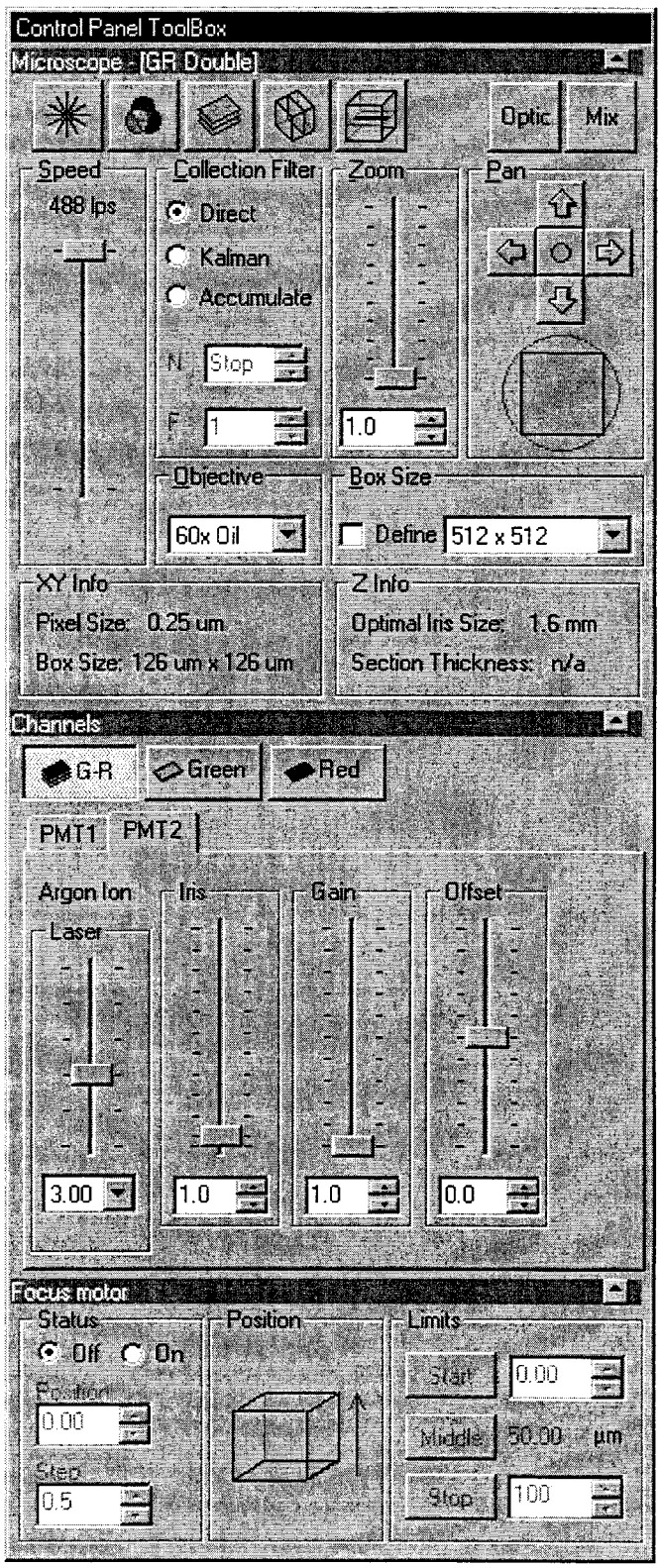


But they can be repositioned in almost any way desired by clicking and dragging the double vertical bar at the left of each toolbar:



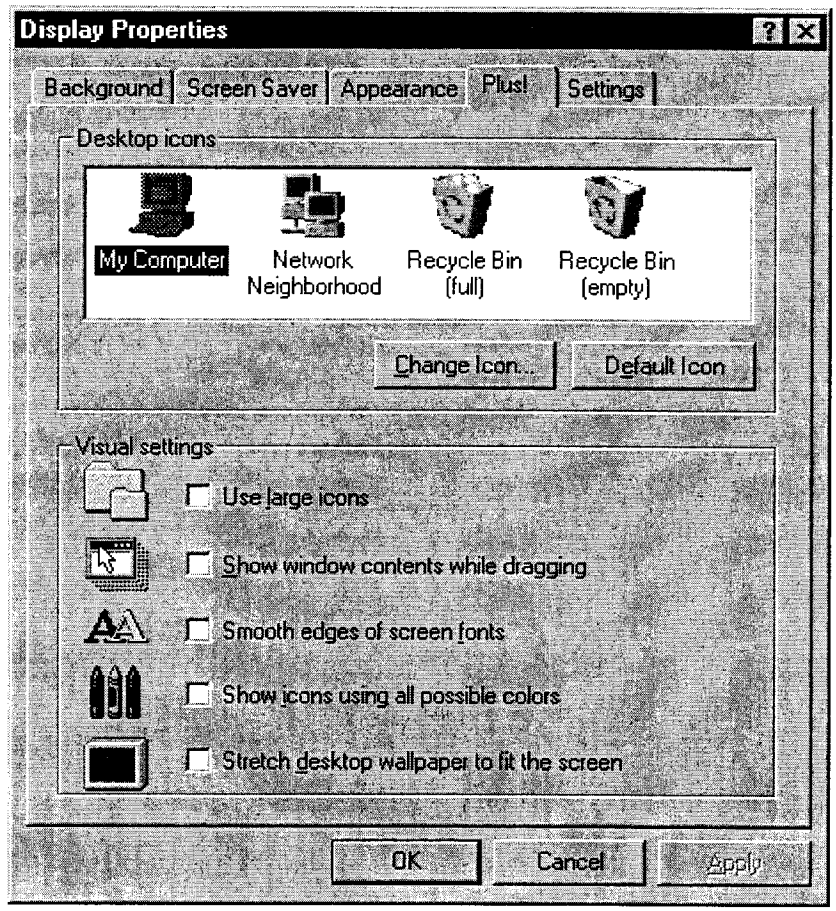
2.1.2 INSTRUMENT CONTROL PANEL

On the righthand side of the screen the instrument control panel will be displayed:



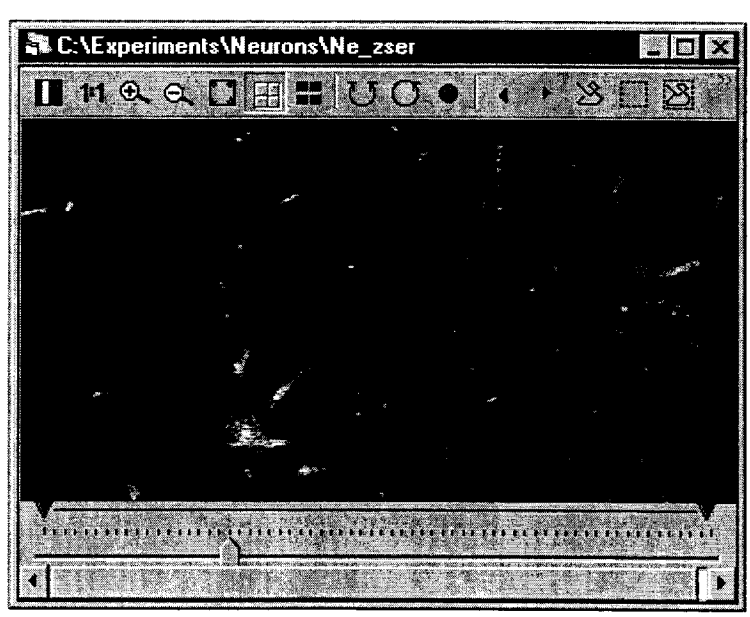
As can be seen, this panel controls the scanning, the detectors and the focus motor. This control panel can be repositioned as desired. To ensure that the window dragging behaves

normally set the Display Properties option 'Show window contents while dragging' to unchecked. (To access this dialog right click on the desktop and select Properties.)



2.1.3 IMAGE DISPLAY WINDOWS

Unlike the OS/2 version it is possible to display more than one image acquisition window at any one time and because LaserSharp2000 is a unified application an image that has been acquired into a window can immediately be processed or operated upon.



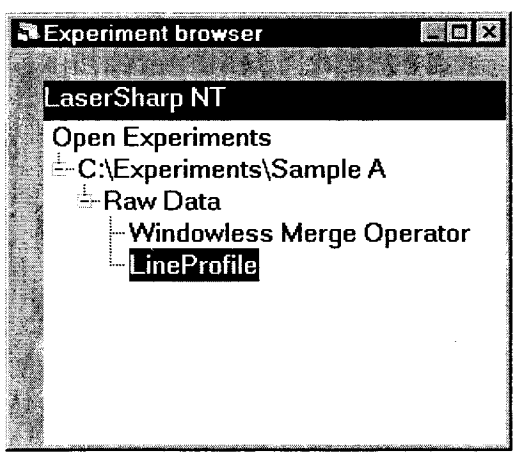
Each image display window has its own display controls in the toolbar and where that window contains a multi-image view an animation slider control appears below the image.

2.1.4 THE EXPERIMENT FOLDER PARADIGM

Perhaps the biggest difference between LaserSharp2000 and the OS/2 version is in the way associated files are automatically arranged in a heirarchical folder structure.

Just as in any application using an MDI (Multi-Document-Interface) it is necessary to create a holder for your data prior to acquiring that data. Using Microsoft Word as an example, you press the New (document) button to create a new document before commencing typing. In LaserSharp2000 you have to press the New Experiment button to create a new image display window before you commence scanning. Of course, if you wish to rescan over a previously collected image then you simply presas the scan button.

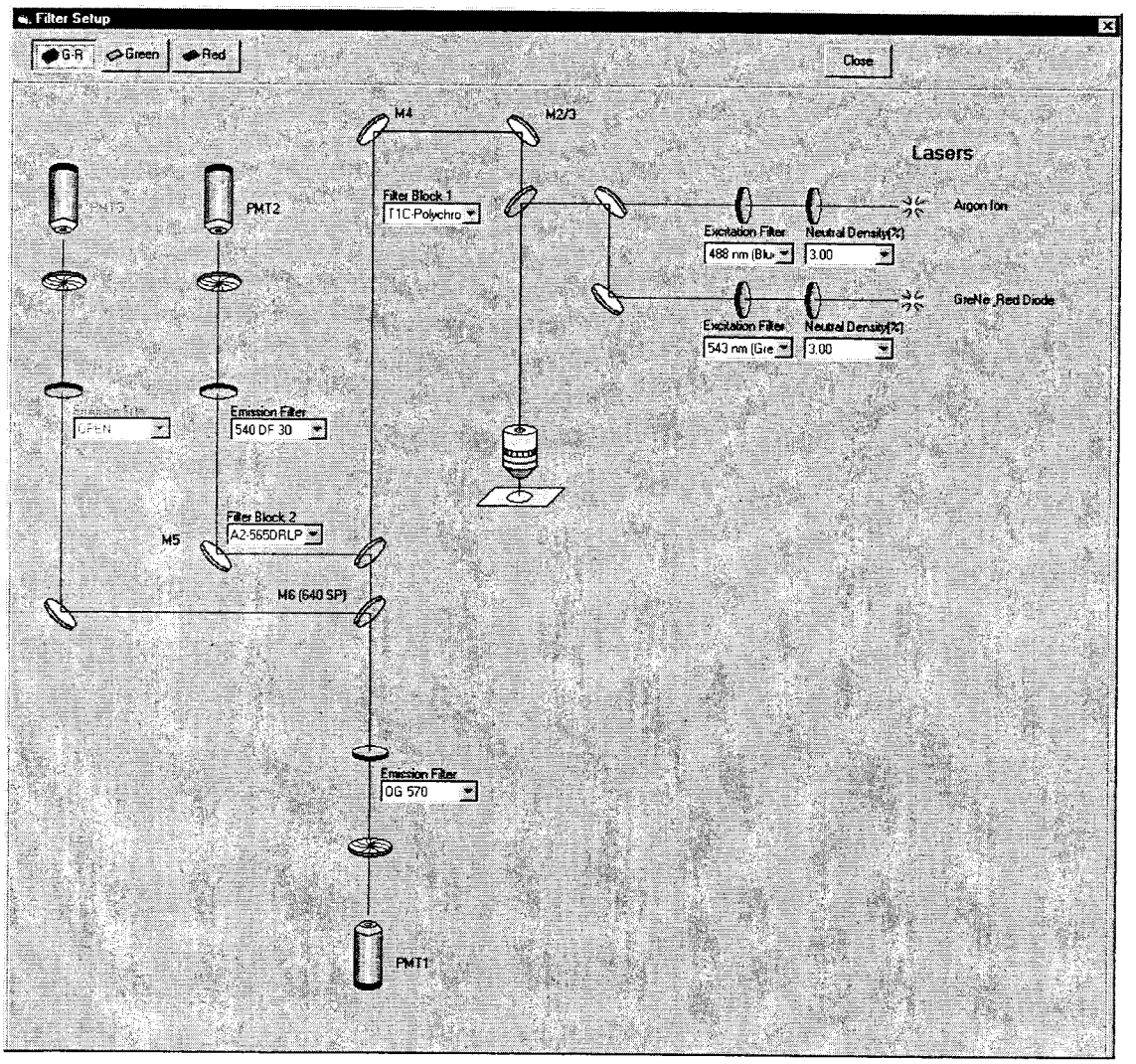
To aid you organising your data, images that are created from the raw data are stored in automatically created and named sub-folders. For example, if you have named an experiment 'Sample A' then a folder will be created called 'Sample A' and your raw data will be placed in a file (called raw.pic) within a sub-folder called 'Raw Data'. If there is more than one channel of data and you have chosen to merge these channels then a sub-folder called 'Windowless Merge Operator' will be created. A sub-folder will be created for each instance of each operation on the data set.



The Experiment Browser allows you to see your open experiments and their respective sub-folders. Note that in each experiment there can only be one Raw Data set.

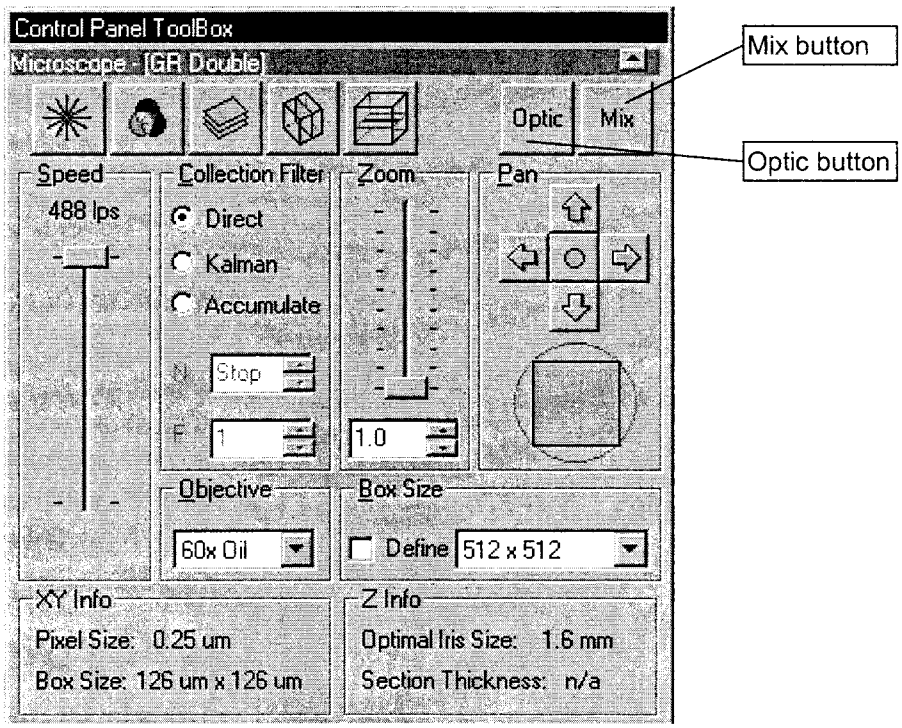
2.1.5 VIEWING AND CHANGING THE SYSTEM OPTICS

Control of the optical system set up is achieved through the Optics/Filter setup configuration screen:



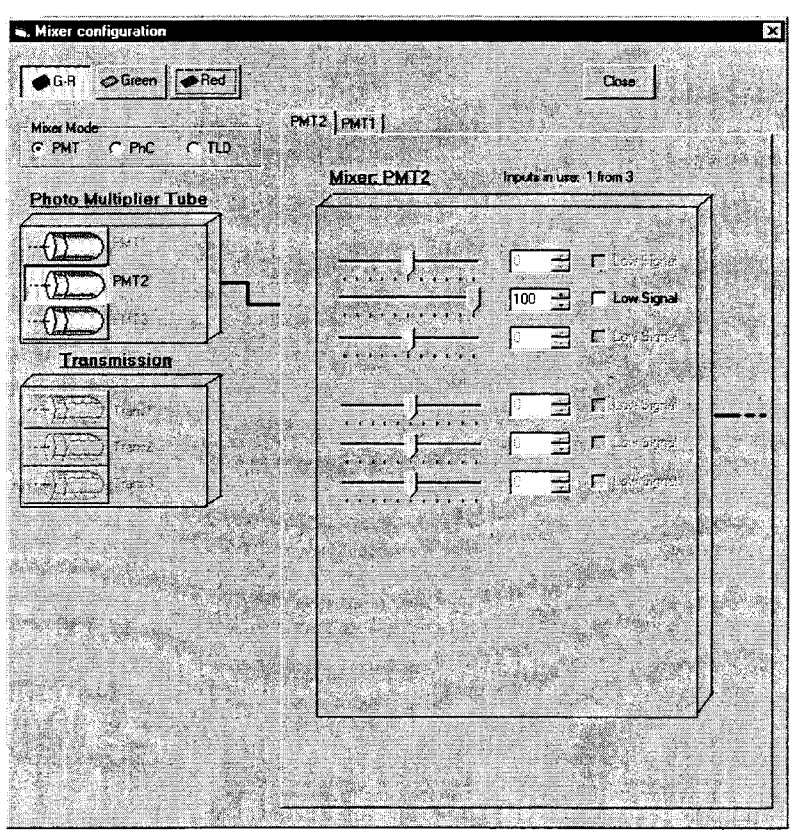
This screen gives a quick graphical view of the system's optical setup and allows you to change emission filters and laser lines.

To show this screen press the 'Optic' button in the Microscope control panel:



2.1.6 MIXERS

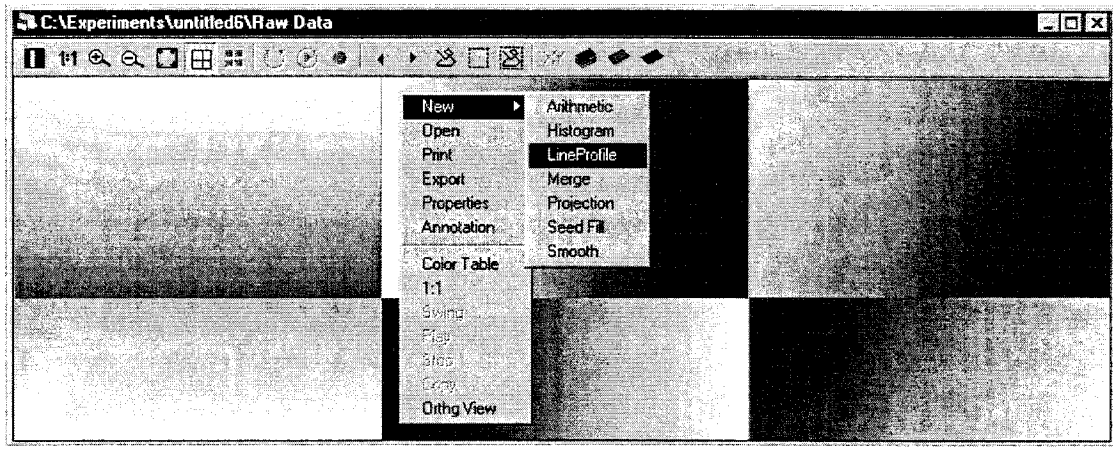
To display the Mixer control dialog press the 'Mix' button.



The Mixer controls enable the additive combination of multiple detectors into one data channel. This contol is very similar to that in the OS/2 version.

2.1.7 POP UP MENU

Image processing and analysis functions are accessed by right clicking on the image to show a pop-up menu:



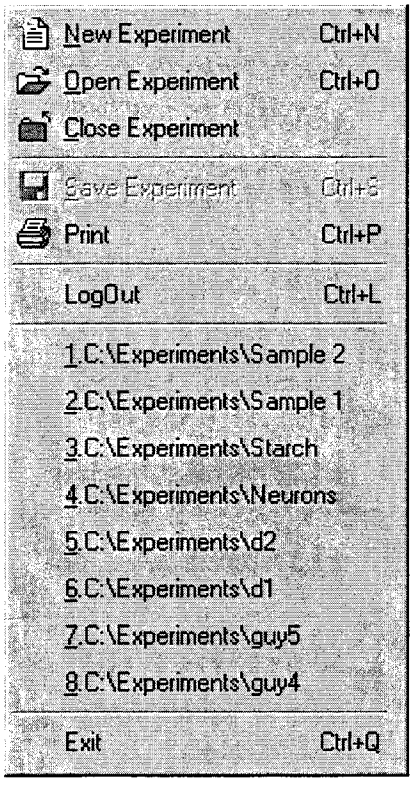
3. SOFTWARE REFERENCE

This chapter provides a reference to the LaserSharp2000 software.

The various components and modes of image acquisition are explained in the following sub-sections.

3.1 Menu bar

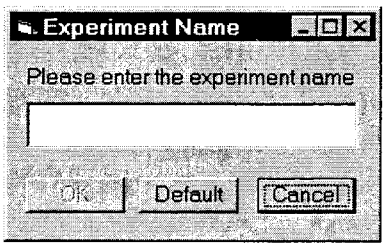
3.1.1 FILE MENU



The File menu contains entries to:

3.1.1.1 Create a New Experiment 

Select this item from the menu or use the button on the application toolbar to create a new experiment. You will be prompted for an experiment name and then an image display window to suit the currently selected Method will be created. If you wish, you can create experiments with a default name (Untitled1,2,3 etc.) by pressing the default button.



3.1.1.2 Open an existing Experiment 

Opens an existing experiment from disk. The experiment will appear in the experiment browser and the desired data can be opened from there. To open a particular component of the Experiment simply double click the item in the Experiment Browser.

3.1.1.3 Close a currently Open Experiment 

Closes the experiment - shuts all windows in the experiment.

3.1.1.4 Save an Experiment

The option Save Experiment As.. is currently disabled but will be implemented in a future version. **Note:** If you wish to rename an Experiment you can do this using the Windows Explorer, but you must ensure that the Experiment has been closed in LaserSharp first.

3.1.1.5 Print an image 

Prints the selected pane from the currently highlighted image. LaserSharp2000 prints to the default printer as determined by Windows NT. For details on setting the default printer see Windows NT Help.

3.1.1.6 Log Out

Logs out the current user and displays the Login dialog ready for the next user.

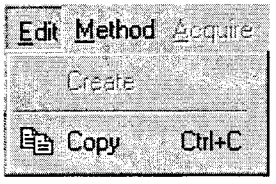
3.1.1.7 A list of recently opened Experiments

The eight most recently used experiments are shown for rapid access.

3.1.1.8 Exit the application

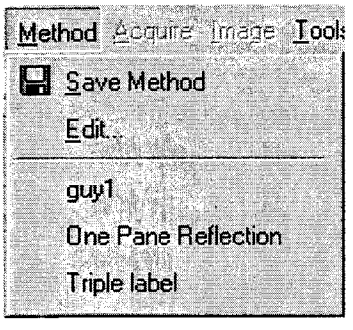
Exits LaserSharp2000.

3.1.2 EDIT MENU



A single entry, copies the currently selected image to the clipboard for transfer to other applications.

3.1.3 METHODS MENU

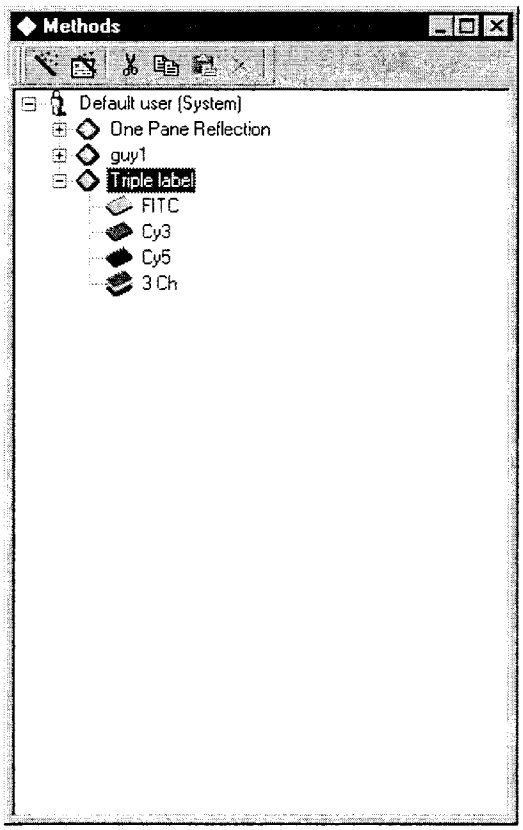


This menu contains entries to Save a modified method and to edit an existing method or to create a new method.

3.1.3.1 Save Method 

Saves the currently loaded method with modifications to the optics or mixer settings.

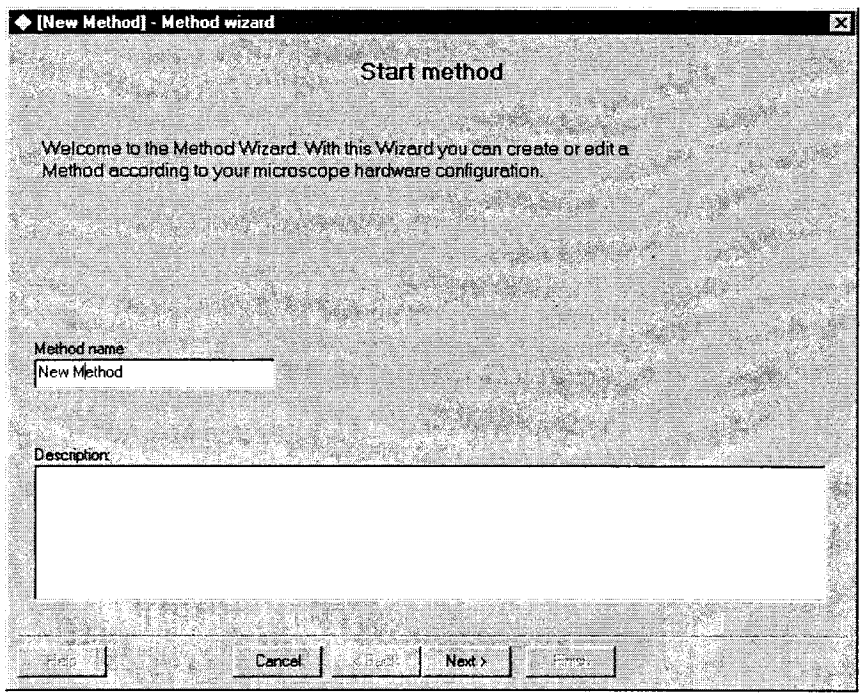
3.1.3.2 Edit... (Method)



The Edit Methods dialog shows a graphical representaion of the methods and their settings on a per user basis. To edit an existing method select the method name in the list and press the Method Editor Wizard button  . To create a new method press the Method Creator Wizard button  . The Method Wizard will take you through the required steps to create a new Method or to edit an existing one.

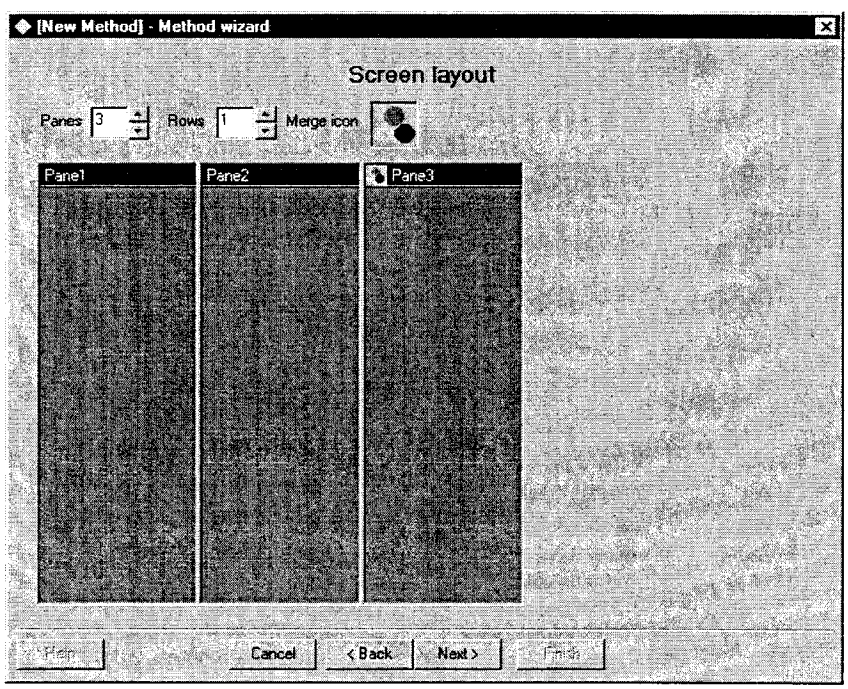
3.1.4 METHOD WIZARD

Step One



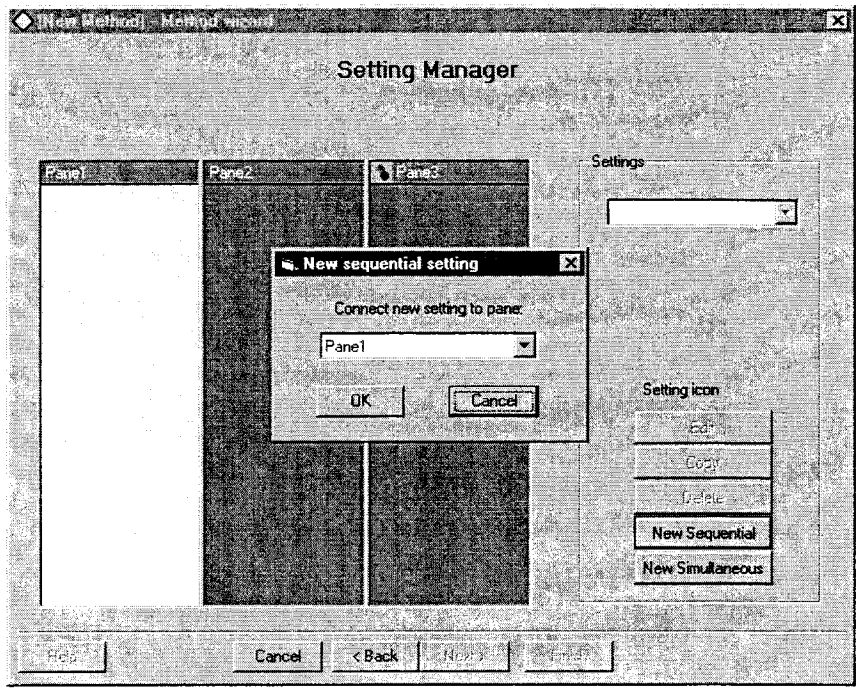
Enter your chosen Method name and description if desired.

Step Two



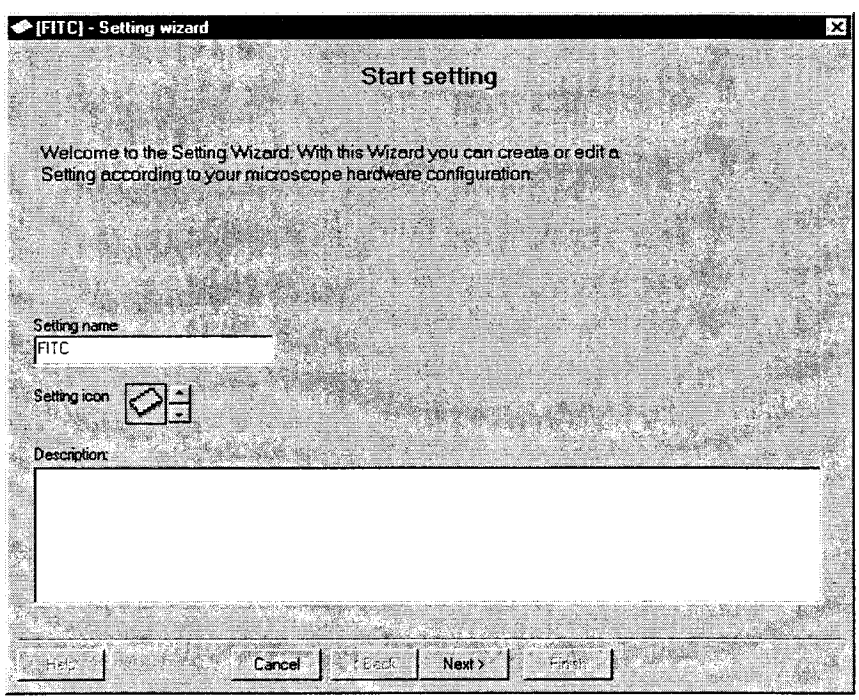
Set up the multi-pane layout by choosing the number of panes and the number of rows. Assign one pane only as the merge pane by dragging and dropping the Merge icon into that pane.

Step Three



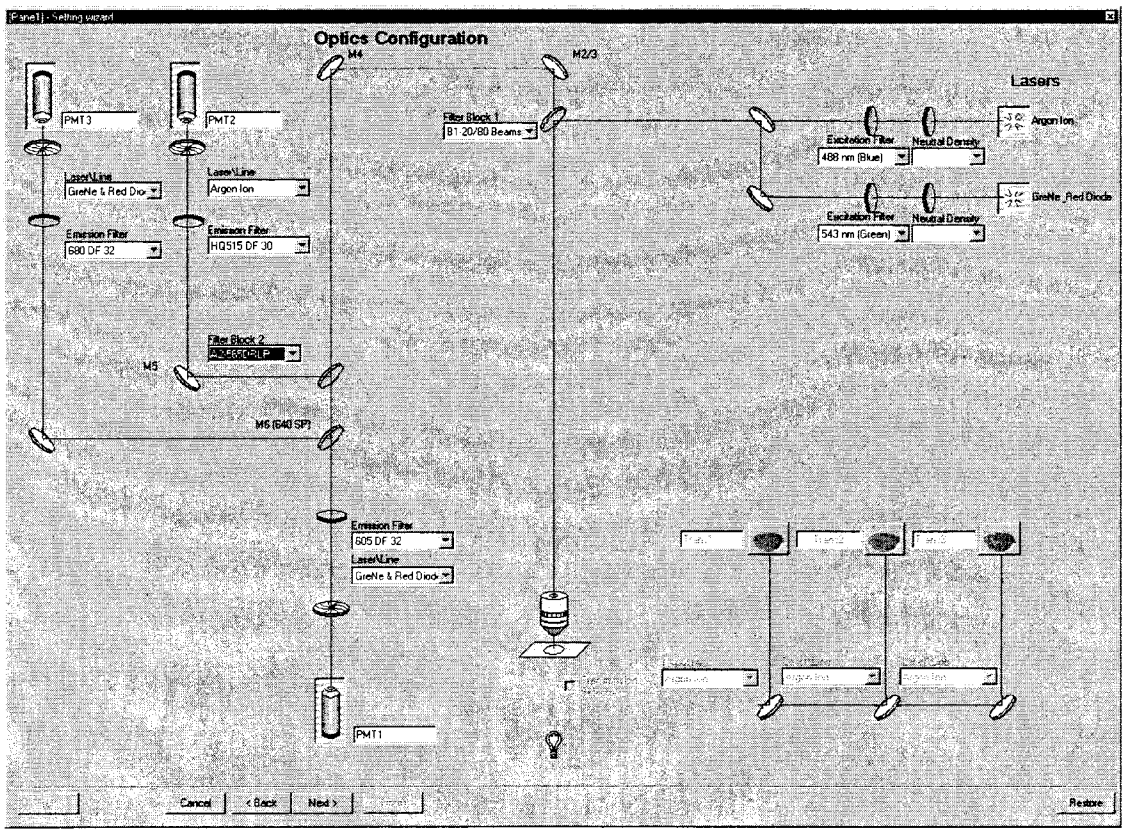
The setting manager allows you to create your Sequential settings (many) and one Simulataneous setting. On pressing the New Sequential setting button you will be prompted to assign that setting to one of the panes (normally you will simply need to accept the default shown).

Step Four



Use the Setting Wizard to define the setting name and the colour of the icon which will appear in the control panel.

Step Five



Set up the instrument appropriately for the setting you desire in the following sequence:

1. Turn on the laser(s) to excite the fluorophore.

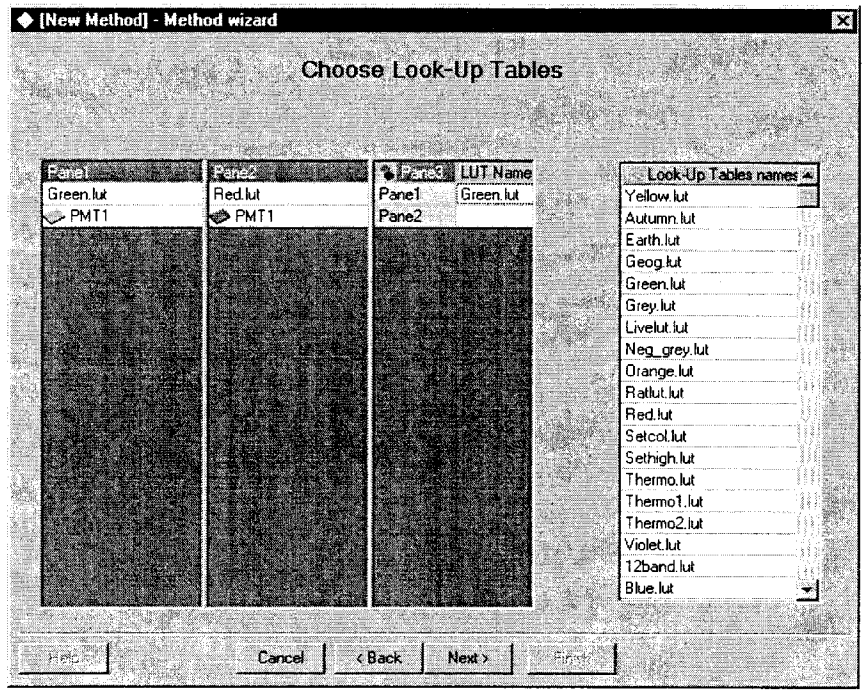
2. Set an appropriate power. This can be adjusted later so don't worry too much about the level set - use less rather than more.

3. Select an appropriate filter block pair to reflect the excitation light to the sample and the emission signal(s) into the chosen PMT(s) and turn on that PMT. Edit the name of the PMT if desired.

4. Select a suitable emission filter for each PMT fom the drop down.

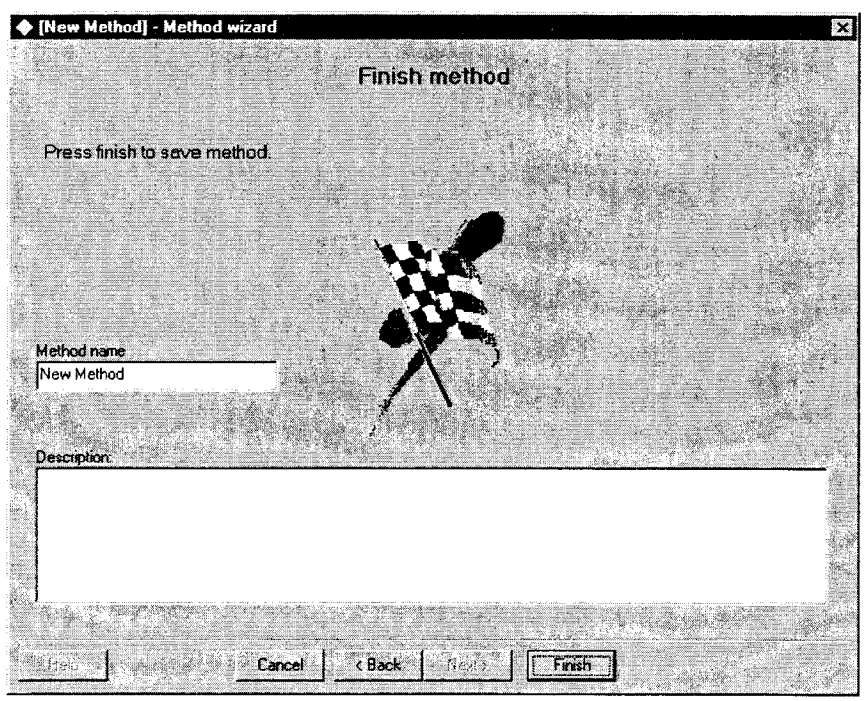
5. For each PMT select the laser line which is causing the fluorescence from the drop down. This operation determines which laser control slider appears in the control panel together with the gain, iris and offset sliders.

Step Six



To define the contents of the Merge pane drag and drop the chosen panes into the Merge pane and then apply the look up tables (LUTs) to the individual panes and to the merged pane.

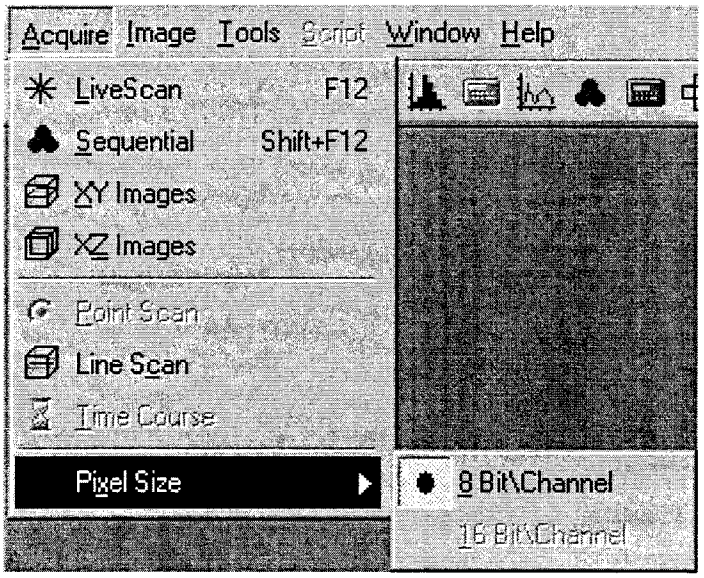
Step Seven



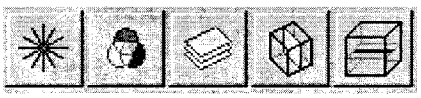
Finish.

Your new method will now appear in the Methods menu and is ready for use. To edit the method use the Wizard and step through to the appropriate stage to make the change.

3.1.5 ACQUIRE MENU



The functionality of the Acquire menu is replicated in the control panel with the collection buttons.



3.1.5.1 Live Scan 

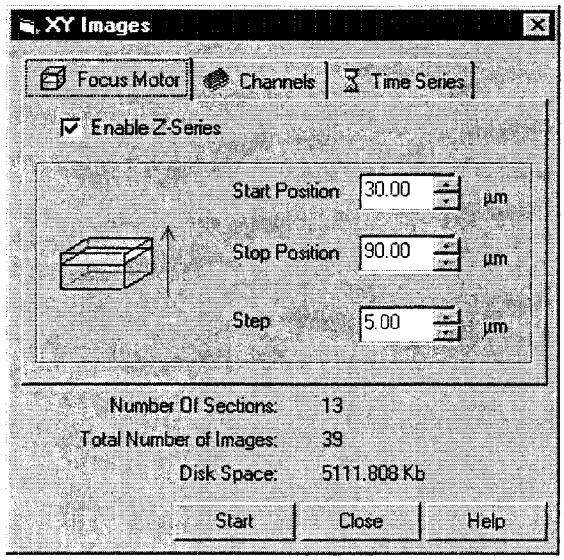
On pressing the live scan button the system will start scanning the galvos and acquiring an image into the active image display window. Whilst the system is scanning almost all system parameters can be changed. For example, detector gain and iris, image zoom, Setting etc.

3.1.5.2 Sequential live scan 

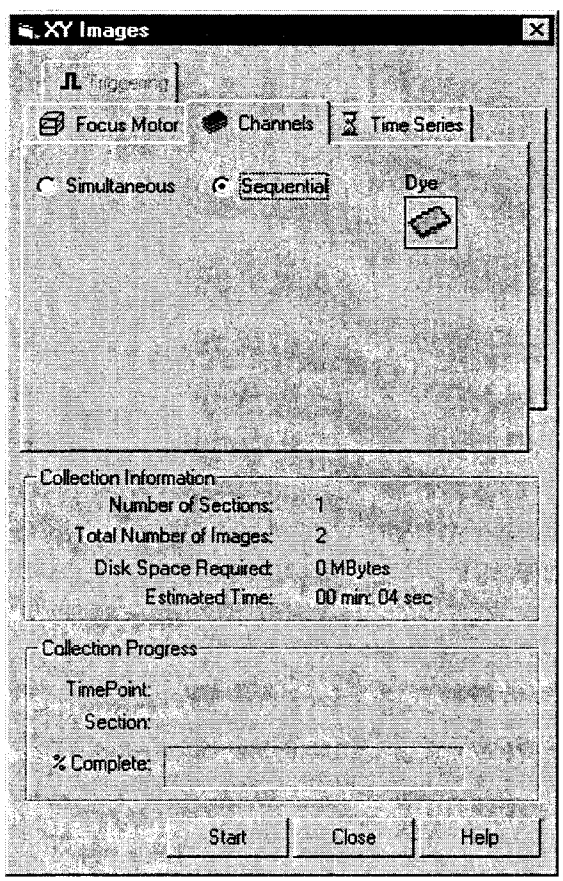
The sequential live scan button automatically cycles through the sequential settings and updates the merge pane (if you have configured one) frame by frame. This mode of scanning is useful when imaging samples which can only be imaged in sequential mode due to bleedthrough.

3.1.5.3 XY (Z-Series) collection 

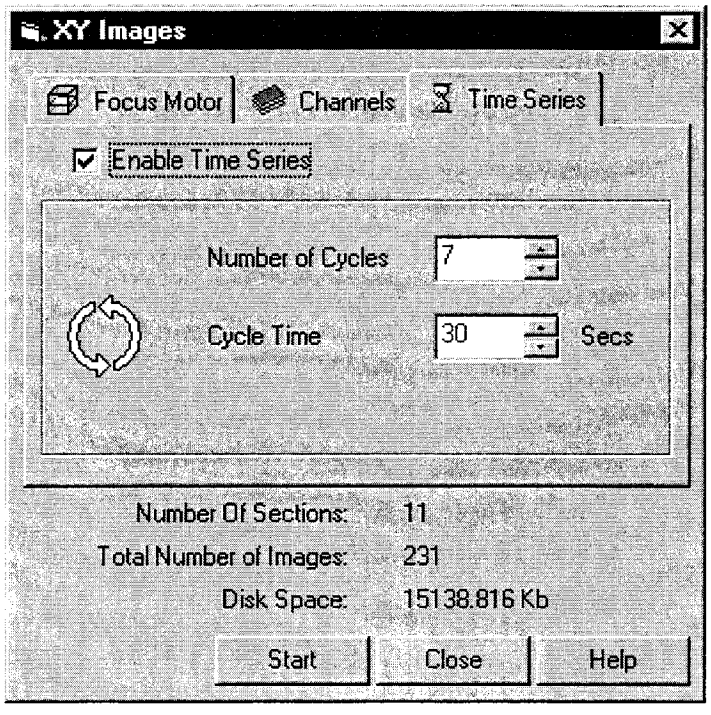
On pressing this button the XY dialog box is shown; Z-Start, Z-Stop and the Z-Step can be reset.



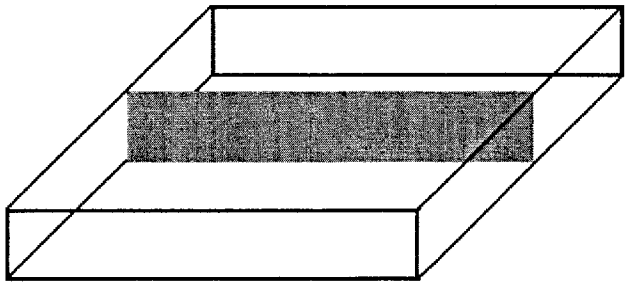
The Channels page is used to select Simultaneous or Sequential acquisition.



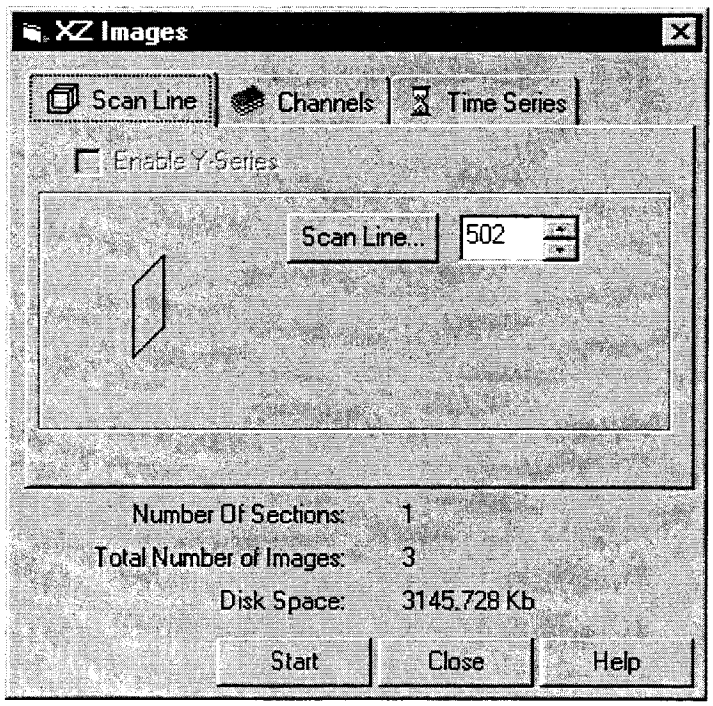
The Time Series page allows either an XY-T series or an XYZ-T series to be acquired.



3.1.5.4 X-Z (vertical section) collection 



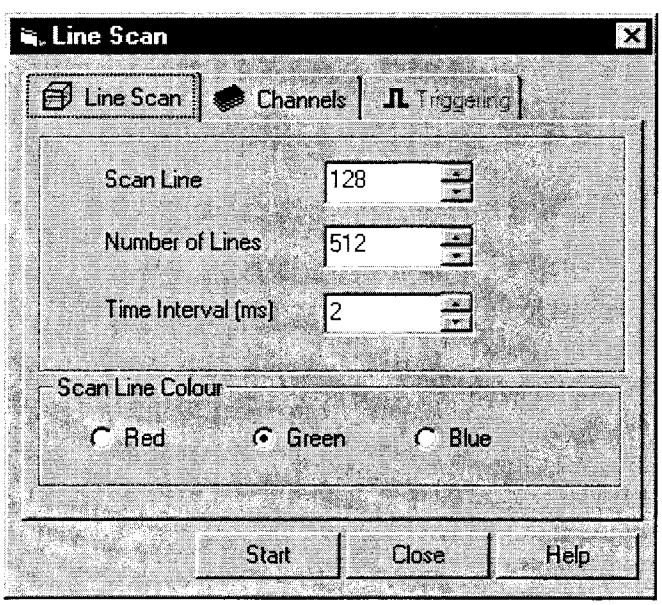
Pressing the XZ button causes the XZ dialog to be shown. The scan line (Y position) is selected using the spin buttons.



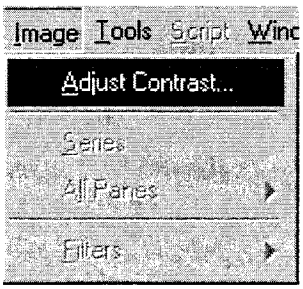
The Channels and Time Series pages function in the same way as they do for XY images.

3.1.5.5 X-T (line scan) collection 

XT data collection runs in two distinct modes will allow collection of upto 1024 lines.

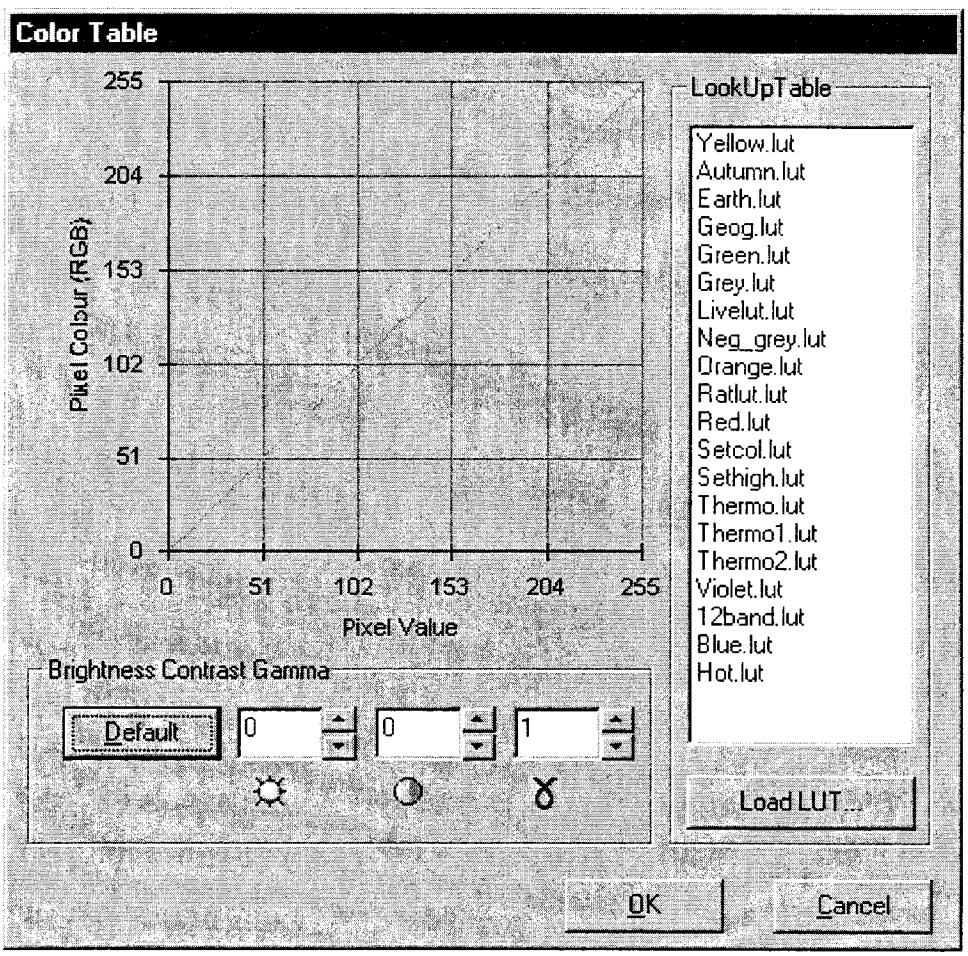


3.1.6 IMAGE MENU



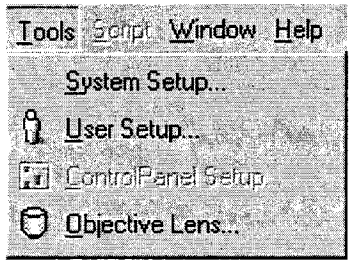
The image menu currently only has one entry - Adjust contrast.

3.1.6.1 Adjust contrast



The adjust contrast dialog allows you to change the brightness, contrast and gamma settings for the current image. You can also load different look up tables from here.

3.1.7 TOOLS MENU

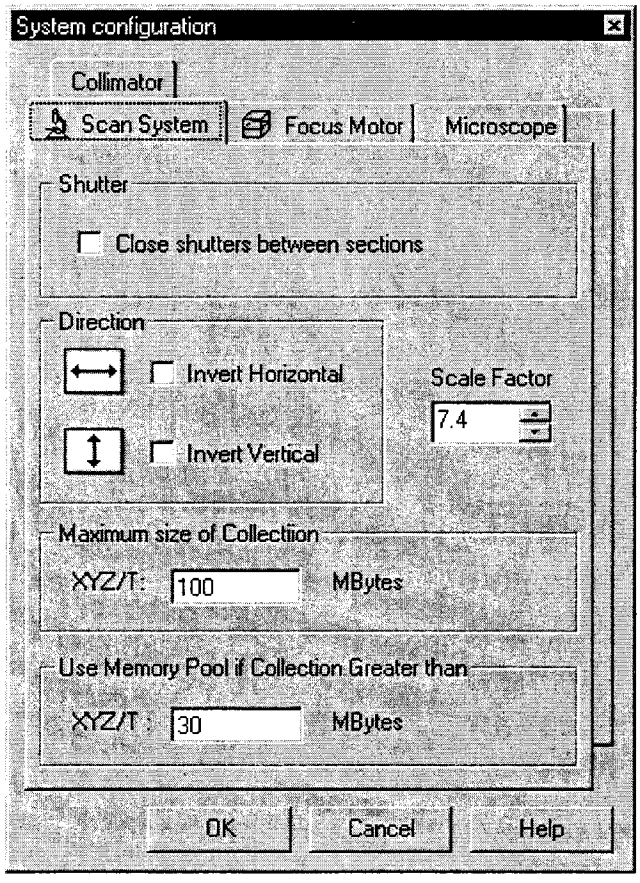


The tools menu contains options for setting up the system configuration, user login details and access rights.

3.1.7.1 System Setup

The system setup dialog consits of four pages.

The first is the scan system setup:



Check the 'Close shutter between sections' to ensure that the beam is turned off between sections. Leave this option unchecked to achieve the fastest possible series acquisition.

The 'Delay to start of scanning' option allows the user to determine the period between the opening of the shutter (or operation of the AOTF) and commencement of scanning.

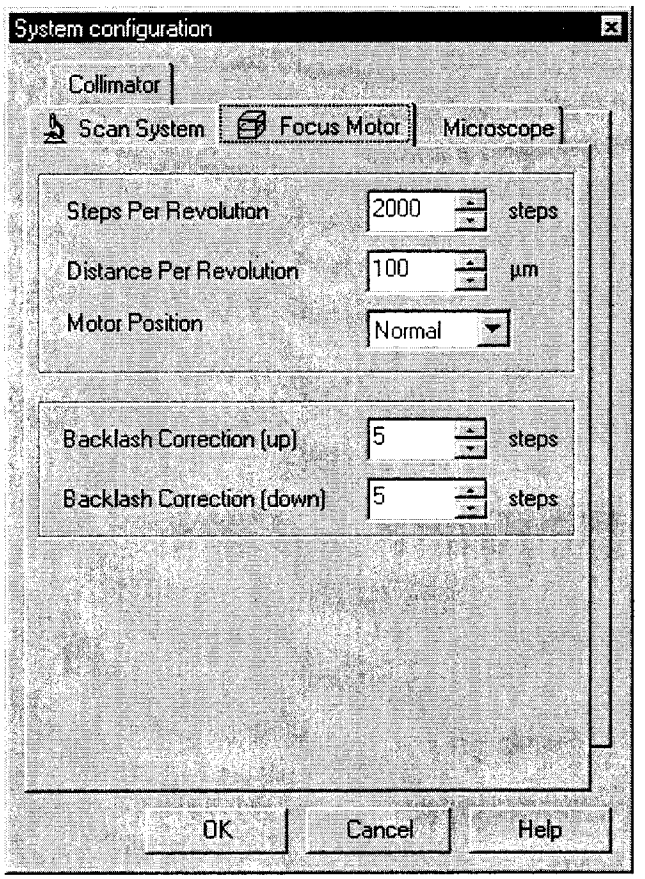
The Direction controls allow you to imvert the direction of scan in both axes to orient the image on the screen to be the same as that seen down the eyepieces.

The scale factor determines the XY calibration of the system and will be set up at the time of installation. **Changing this will render measurements incorrect.**

The 'Maximum size of collection' value should be set to a size such that the real memory and virtual memory of the PC cannot be exceeded.

'Use memory pool if collection is greater than' should be set to a value that is lower than the amount of real memory remaining available.

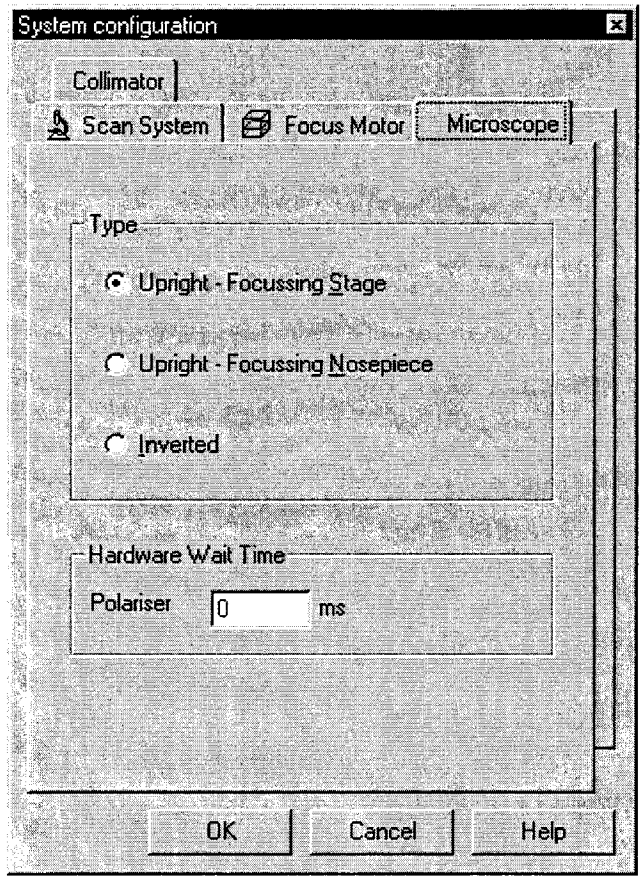
The second page is for set up of the focus motor:



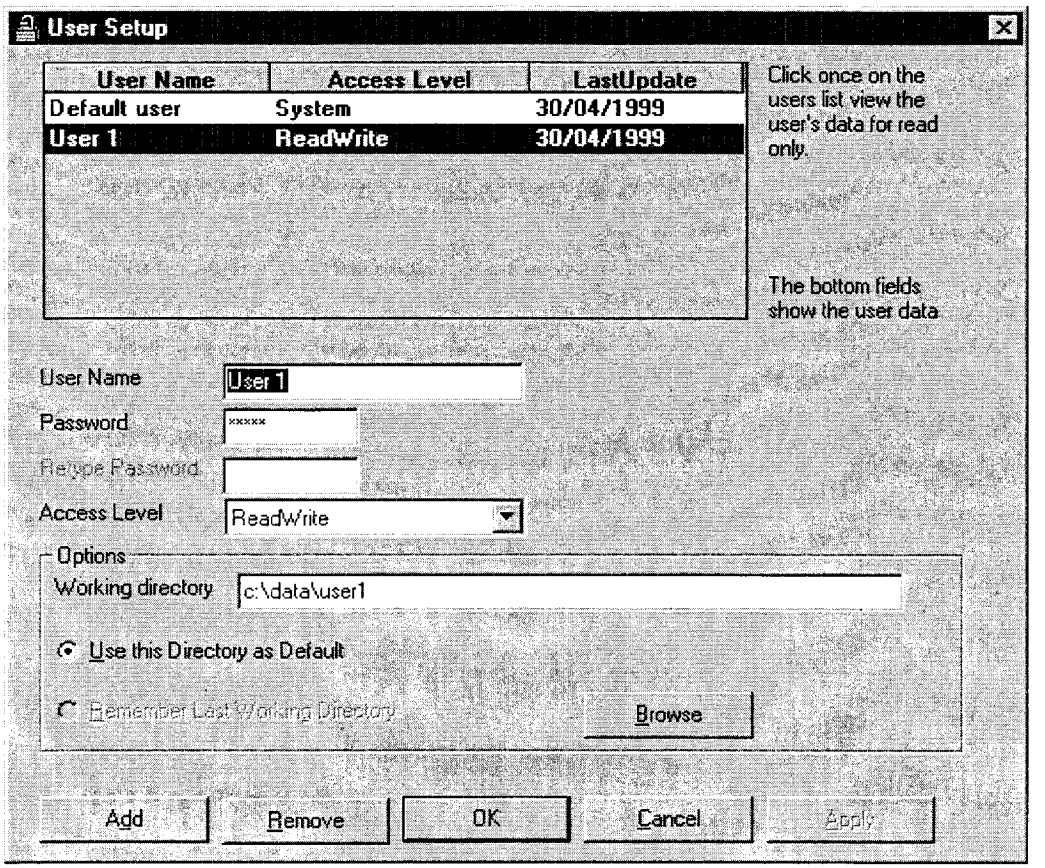
Again, all of these settings will be made at installation and will only need changing if you change the microscope to which your system is attached.

The Collimator page can be ignored as this applies only to Radiance multi-photon systems.

The Microscope page determines how the Z-Focus icon operates dependent upon the type of microscope you are using. The hardware wait time should be set to zero for MRC-1024 systems.


System configuration
Collimator
Scan System
Focus Motor
Microscope
Type
Upright - Focussing Stage
Upright - Focussing Nosepiece
Inverted
Hardware Wait Time
Polariser
0
ms
OK
Cancel
Help

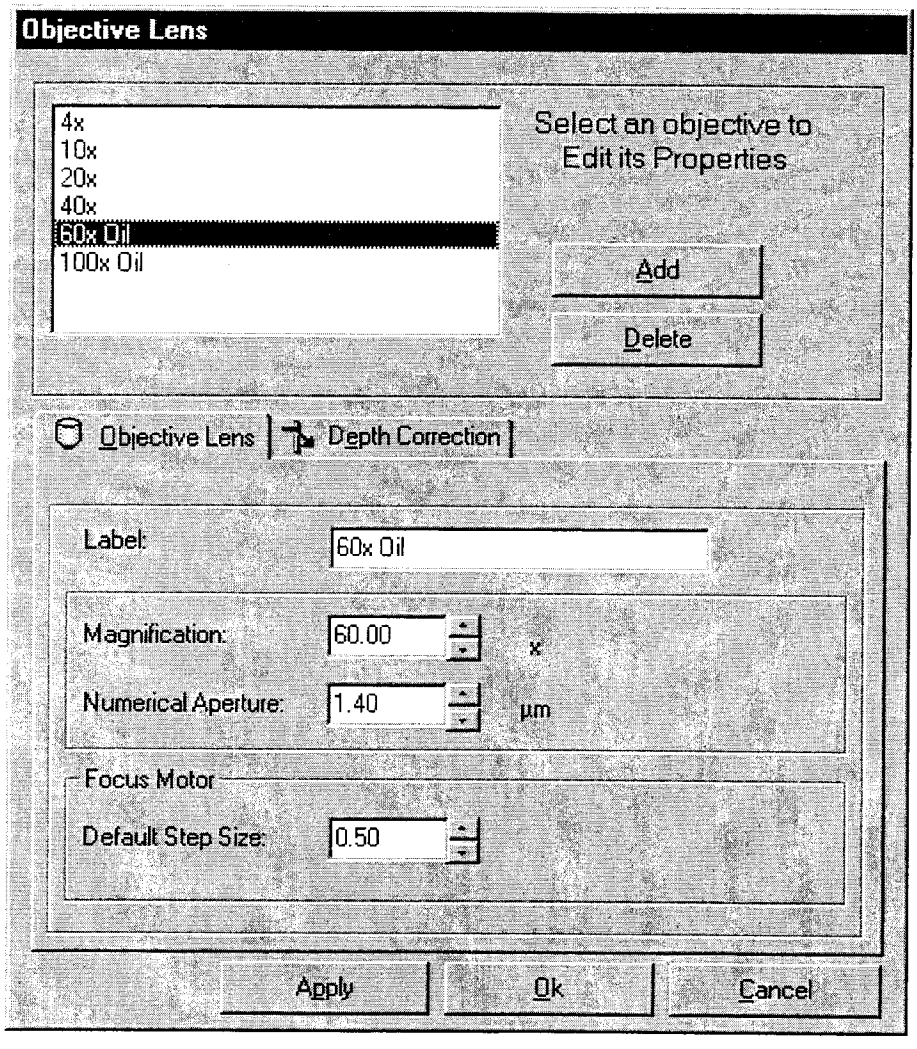
3.1.7.2 User set up



The User setup dialog allows authorised users to add or delete other users or to edit their access rights. There are three access levels; System, Read/Write and Read Only. It is recommended that the appointed system manager has System rights, all authorised users should have Read/Write access (so that key system configurations cannot be accidentally modified) and that unauthorised users are give Read Only access. Only users with System access can create, delete or edit other users or edit their access level.

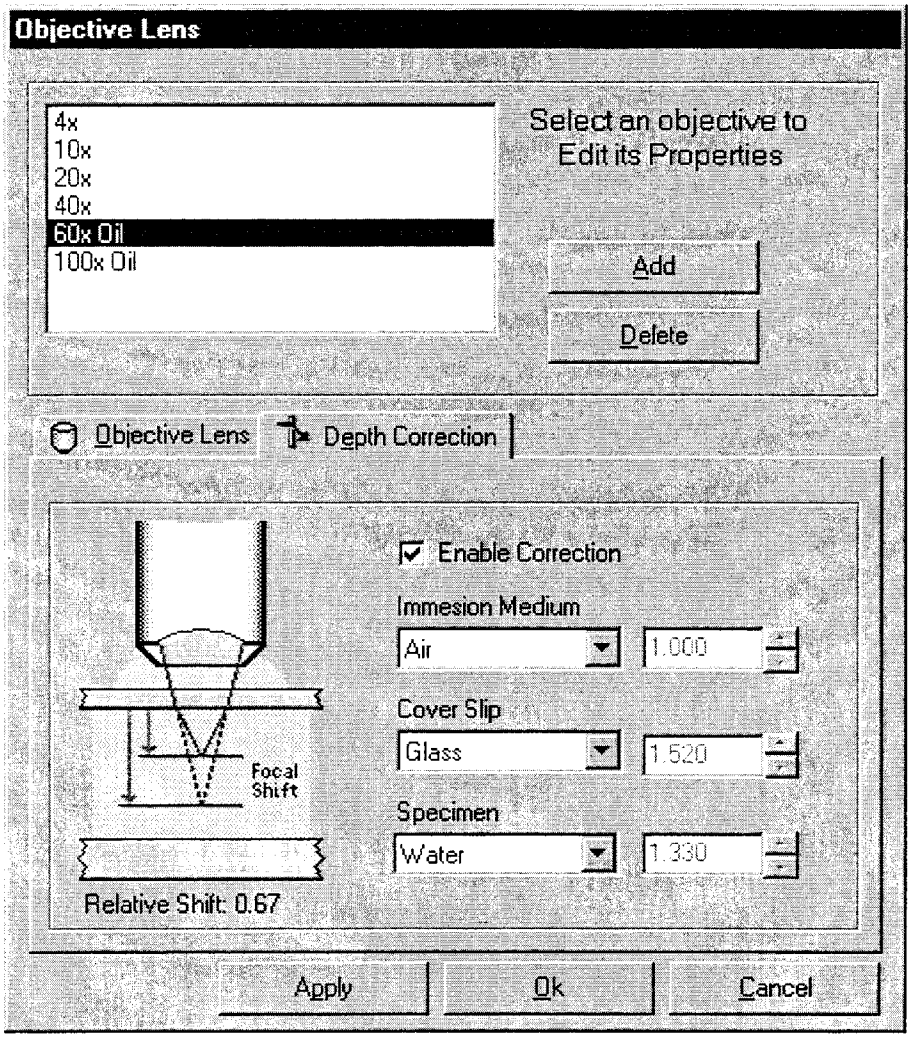
A default working directory can be assigned for each user so that their data is automatically saved in a known folder on the system.

3.1.7.3 Objective lens set up

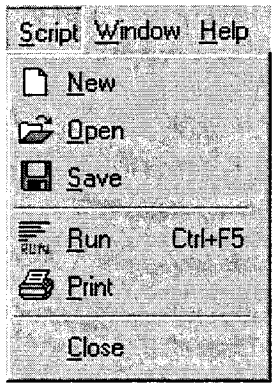


The Objective lens page of this dialog allows you to add, edit or delete lens descriptions from the list which appears in the control panel. The Magnification value is used for calibration of measurements. The default focus motor step size should be set to a convenient value for each lens.

The Depth Correction page activates a correction algorithm to correct for axial geometric distortion caused by mismatching of refractive indices above and below the cover slip.


Objective Lens
4x
10x
20x
40x
60x Oil
100x Oil
Select an objective to Edit its Properties
Add
Delete
Objective Lens
Depth Correction
Focal Shift
Enable Correction
Immesion Medium
Air
1.000
Cover Slip
Glass
1.520
Specimen
Water
1.330
Relative Shift: 0.67
Apply
Ok
Cancel

3.1.8 SCRIPT MENU



The script menu contains entries for editing and running scripts. These scripts are standard Microsoft Visual Basic Scripts (VB Script) and their documentation should be consulted for details of this programming language.

Scriptable functionality within LaserSharp2000 which is available at release of V3.0 is:

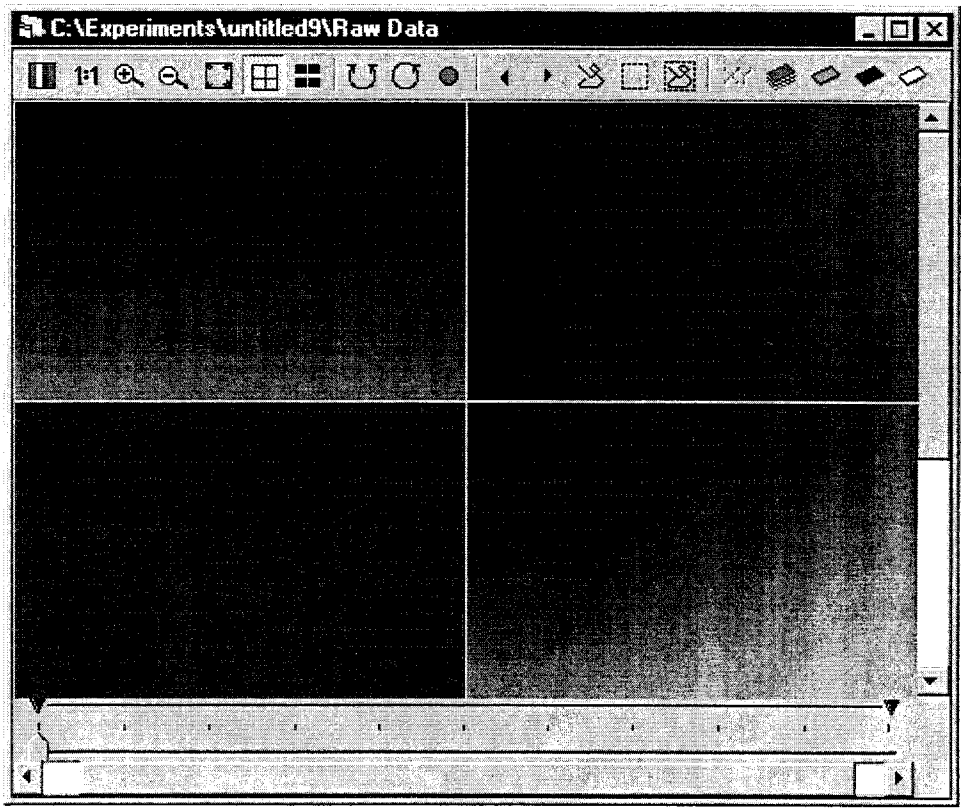
Instrument Control
- Start a scan series (currently XY scan only)
- Detector gain, iris, offset
- Laser attenuation (power)
- ZMotor position, move, switch on/off
- Setting selection (within given a method)

Application Access
- Current scanned 2D images
- Windowless image processing operation (yet to be fully implemented)

Confocal data manipulation and access
- Creation of multi-dimensional confocal image matrix
- Initialise and allocate image buffers for image matrix
- Access/manipulation of dimension information
- Access/manipulation of Calibration information
- Access/manipulation of individual 2D images and their individual pixels
- Fast memory copy between 2D images

Scriptable Status Bar
- Write text to the status bar from within a script

3.2 Image display window



Each image display window houses its own set of controls:

Apply LUT

Set zoom to 1

Increase zoom

Decrease zoom

Toggle on/off Fit toview mode

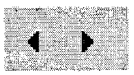
Toggle between single and multi-pane view. can also be achieved by double clicking the image panes

Show montage display of data

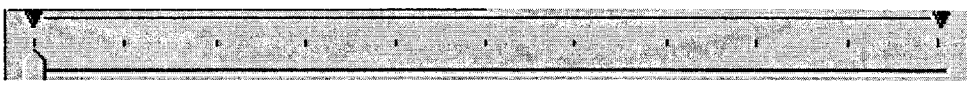
Rocking animation of multi-image data set

Looping animation of multi-image data set

Stop animation



Increase/decrease animation speed



The slider at the bottom of multi-image files can be used to manually animate the images. The Small black pointers at the top and bottom are used to set the extent of the animation.

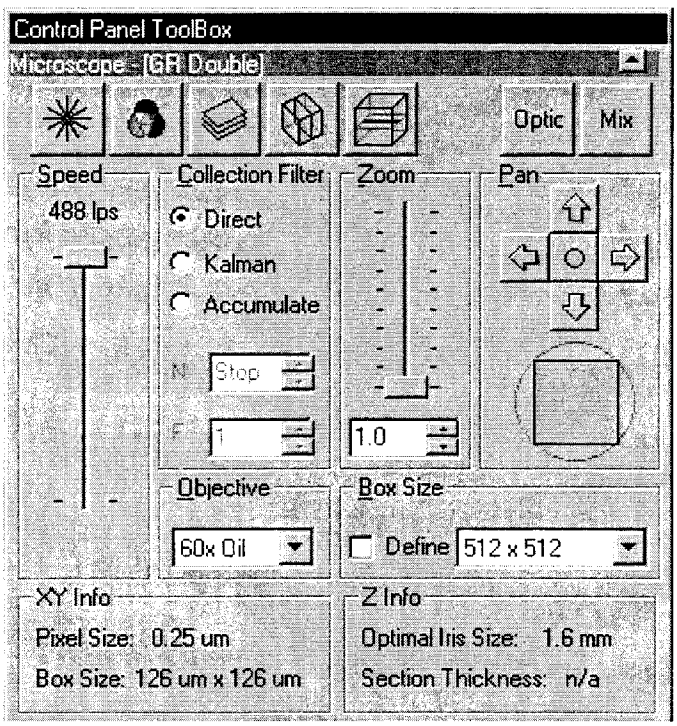
Note that the iamge can be displayed in 'full screen' mode by pressing the  button. To return to 'windowed' mode press escape.

3.3 System control panels

This section describes the control panels on the right-hand side of the screen which are used to control the microscope. The panel is composed of three sections.

All of these panels can be shown or hidden using the  button.

3.3.1 MICROSCOPE CONTROL



Speed

The scan speed has a range of possible settings. For MRC-1024 systems the line frequency has two possible settings: 500 Hz or 166 Hz.

In addition to controlling the line scan frequency one can separately modify the frame rate:

Normal

At the Normal speed if the line (or X) galvanometer is set at 500Hz it takes about 1 second to acquire a 512x512 image.

Collection filters

In the following sections:

P_n is the new pixel value

P_{n-1} is the previous pixel value
I is the input value
n is the current frame number
f is the factor

Direct

This is the default setting. The contents of the image window(s) is overwritten with the new data. The 'factor' value is ignored, and the entry boxes for this value are disabled in this filter mode.

$$P_n = I$$

Kalman

With this filter, the pixel values are calculated:

$$P_n = I/n + P_{n-1} (1 - {}^1/_n)$$

This filter enables a display of the average of all the frames since filtering was started. Full intensity is always maintained, but the signal to noise ratio increases.

If collection is stopped and then started, the image is cleared before restarting.

The "factor" value is ignored, and the entry box for this value is disabled in this filter mode. When the user stops scanning in this mode, the filter selection returns to "Direct" and N is set to zero.

Accumulate

In accumulate filter mode 'N' frames are accumulated (added).

The resultant values are scaled by 1/Factor. If 'Factor' and 'N' are set to the same value, then the final result will be the same as if Kalman filtering had been used. The image is not cleared automatically before scanning in the accumulate mode, so it is necessary for the user to do this using the eraser button. Accumulate to 'N' can be used to produce brighter images, and can be set prior to the collection of a multi-image file like a z-series.

Before doing this, it is worth checking that image saturation will not occur and that N is not set too high. Saturation occurs when so many pixels within a structure attain a value of 255 that the greyscale information is lost. To combine Accumulation of signal with averaging, set a ratio of N:F where the greater N is, the better the averaging, and where the N:F ratio determines how many times brighter the eventual image will be compared to a single 'Direct' scan. For instance, if the original image needs to be 5 times brighter and averaged, N can be set to 30 and F to 6 so the image is averaged over 30 frames. Because the pixel values in each frame are divided by a factor of 6, the final image is 30:6 or 5 times the original intensity.

Accumulate to peak

Accumulate to peak is currently not available in LaserSharp2000.

Factor (F)

This value is only used by the Accumulate filter. It is described above.

Number of scans (N)

This is the number of scans to perform. If it is set to zero, then the instrument will continue scanning until the laser button is pressed again.

Objective

This is the magnification of the objective lens on the microscope. This value is used to calculate distances on an image. Users must remember to change this value to match the lens being used in order to collect calibrated data.

Zoom

Changing this value controls the amplitude of the angle through which the galvanometer mirrors scan, and hence the area of the sample that is scanned.

The value in the edit box is a magnification value. This is a true optical zoom. An optimum value associated with each lens is shown in the table on the next page, which can be used as a guide. Exceeding these values will make the image bigger but with no further improvement in resolution (empty magnification). Moreover, any fluorochrome bleaching will be accelerated because a constant flux of laser light will be concentrated into a much smaller area. Of course, zooming can be used to deliberately apply a high irradiation dose to a small area for applications such as FRAP or caged compound release.

Pan

Press the appropriate arrow to move the scanned area in the required direction. The central button returns the scanned to region to the centre or 'home' position.

The bitmap at the bottom right of this window indicates how much of the image is being shown. The outer ring is constant, and represents the full field of view of the objective lens.

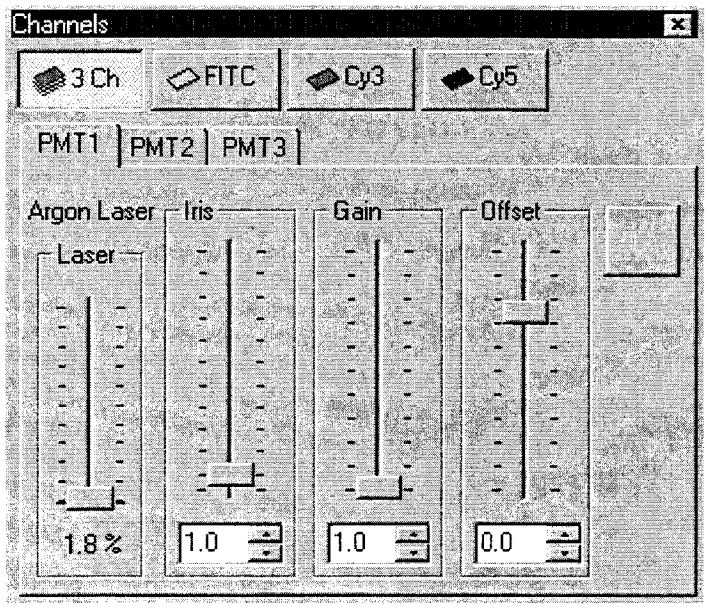
The rectangular border represents the scanned region. The shape of this will depend on the box size chosen. As the zoom factor is increased above 1.0 this rectangle will become smaller.

The solid rectangle represents the region that can be seen on the screen. When the box size is, for example, 256 by 256, the solid rectangle will fill up the border because all of the image can be seen. When, for example, the box size is 1024 by 1024, and the system is in quad mode, then not all of the acquired image can be seen. The solid rectangle will indicate this.

Box size

The box size determines the resolution of the acquired image. A user defined box can be set by checking the 'Define' box.

3.3.2 CHANNELS CONTROL



The appearance of this panel is determined by the currently selected Method. All the controls for a particular channel are grouped on a single page within the panel.

The **Laser** slider controls the intensity of laser light delivered to the sample. If an AOTF is fitted then this is a continuous control, but it moves in steps for a system with ND filters.

The **Iris** control allows the user to select an iris diameter from 0.8mm to 12.0mm in 0.1mm steps. The iris is a variable iris diaphragm, which acts as the confocal aperture in front of each PMT.

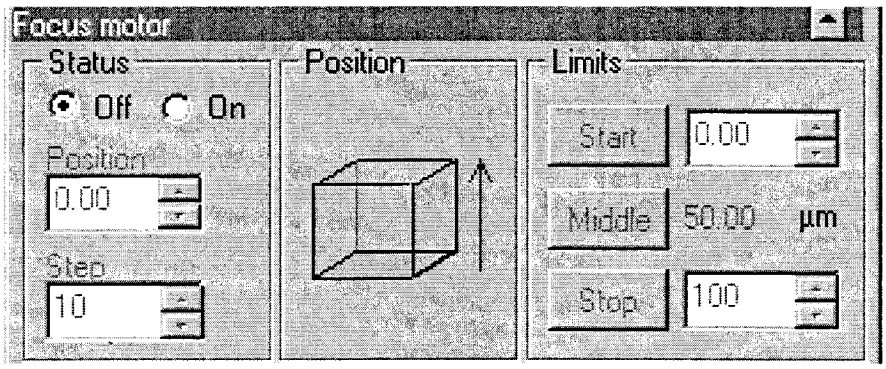
The **Gain** control allows the user to select a value from 0 to 100 in steps of 1.

The gain is (non-linearly) proportional to the voltage applied across the PMT's dynode chain. Increasing the gain will produce a brighter image but will also increase noise in the image. This noise can be reduced by Kalman averaging as described previously. If the gain is set too high, the image may be saturated, that is, too many pixels are at peak intensity.

The **Black level** control allows the user to select a value from -199 to 200 in steps of 1. The default value is 0.

The black level sets a DC offset on the electronic signal. The offset should normally be set to a value close to zero. Precise setting of this value is best achieved using the SETCOL look-up table (LUT) as decribed in Tutorial 1. In some circumstances the offset can be used together with the gain control to enhance an image with low contrast. An example of such an instance is in transmitted light imaging using phase contrast optics. The contrast mechanism of phase contrast imaging generates an image with positive and negative intensity deviations from a mid-grey background. Caution should used when setting the black level - too high a setting will compress the effective dynamic range of the detctors and a too low a setting will exclude low intensity information from the image (clipping).

3.3.3 FOCUS MOTOR CONTROL



This portion of the microscope control panel only appears if the z-drive system is connected and active when the software is initialised. The commands in this panel control the stepper motor which is attached to either the microscope fine focus knob or in the case of Zeiss microscopes to the coarse focus knob. The z-position is specified in microns. The motor has a resolution of 2000 increments per revolution. This translates into a minimum stage or lens movement (resolution) of 0.05 microns.

On/Off

This control determines whether the stepper motor coils are powered. None of the other commands in this panel have any effect upon z-position unless the motor coils are powered. You may notice that the fine focus control offers considerable resistance and becomes diffficult to turn when the coils are powered. It is inadvisable to make a habit of turning the fine focus by hand when the motor is on, as this could lead to wear in the linkage between the motor and the focus knob. Whenever the motor is switched to from On to Off, the Position value is reset to zero. However, if a value of zero is entered while the focus motor is ON, the stage will move to the zero position relative to its current position.

The focusing drive motor has 2000 increments per revolution. On Nikon, Olympus and Leica microscopes, this corresponds to 20 increments per micron of travel, and thus a minimum vertical movement of 0.05 microns. In the case of the Zeiss microscopes an 18:1 gear box is used to drive the coarse focus and a minimum movement of 0.05 is achieved. For all microscopes, motor operation will be calibrated by the Bio-Rad installation engineer, using the **Tools** drop-down menu.

Position

This value changes by an increment determined by the Z-step each time one of the arrows is pressed, either with the mouse or by pressing the function keys F7 and F8 on the keyboard. An arbitrary z-position can also be entered directly. Whenever the motor is switched to from ON to OFF, the **Position** value is reset to zero. Changing the **Position** affects the z position icon if it is displayed.

Step

This value sets the distance from one Z-level to the next. A recommendation is provided, based on the optical sectioning capability of the currently specified microscope objective, determined by its numerical aperture.

The recommendation is for a lower limit. As a rule of thumb, it is best to use a Z-step which is one-half the vertical spacing between the features to be resolved.

Start

This determines the starting level for Z-series and Vertical sections. Clicking on the button loads the current position into the Z-Start value.

Stop

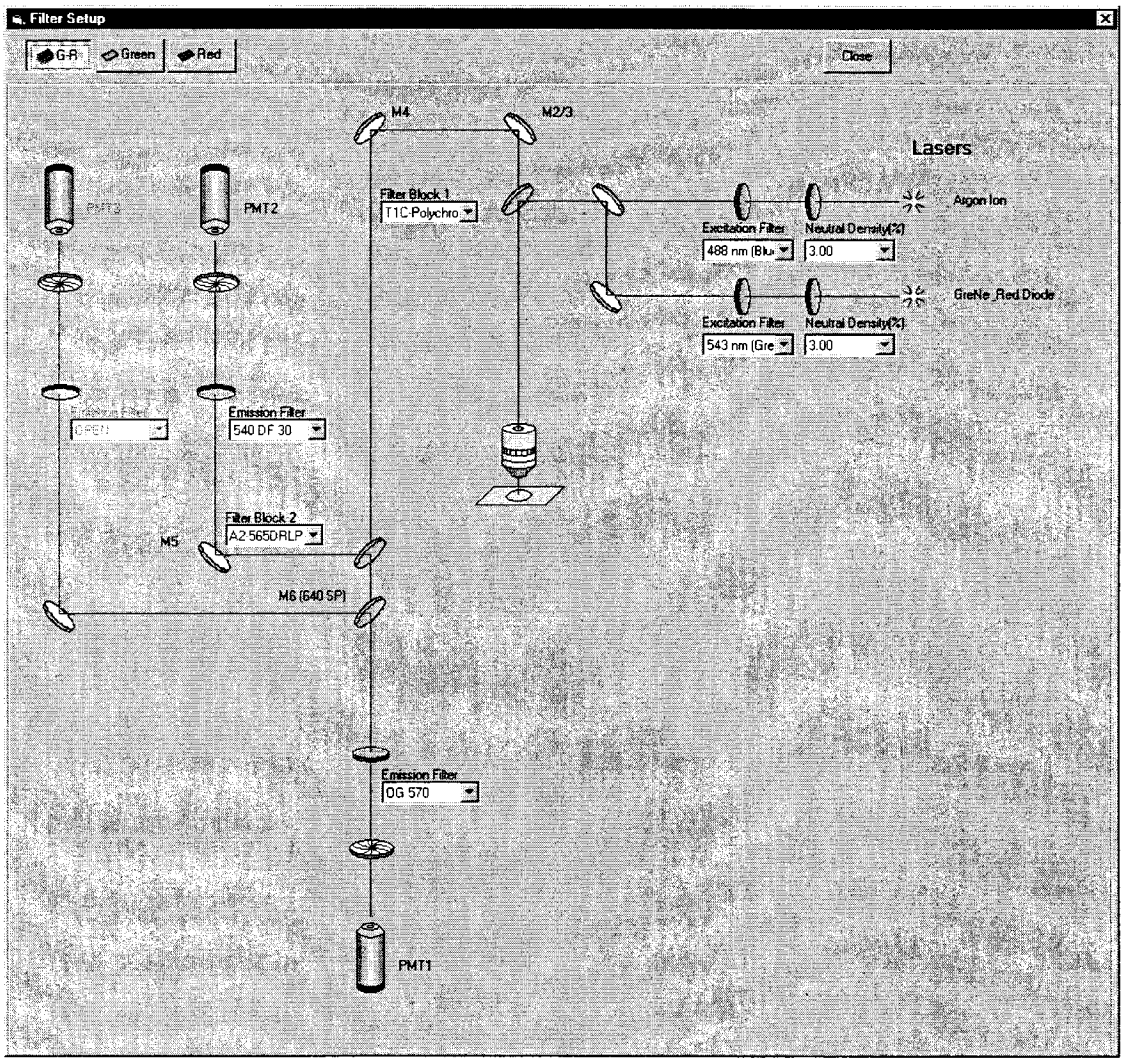
This determines the stopping level for Z-series and Vertical sections. Clicking on the button loads the current position into the Z-Stop value.

Middle

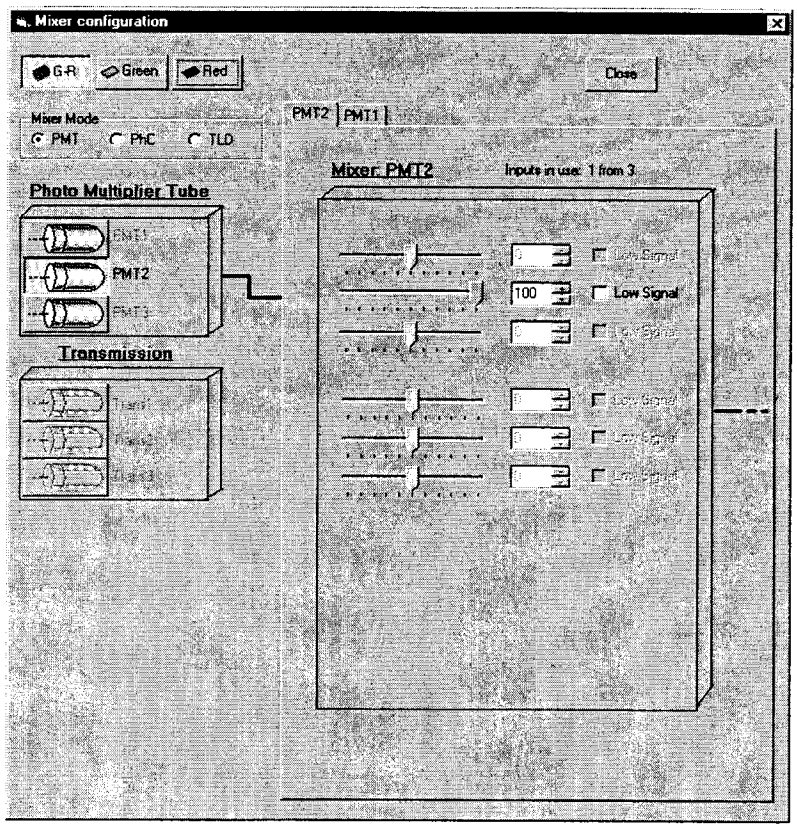
Pressing this button moves the focus to the mid point between the Start and Stop positions.

3.3.4 OPTICS PANEL

The optics panel gives a graphical representation of the systems current configuration and allows this to be modified as desired. Filters and laser power settings can be freely changed whilst the system is scanning.

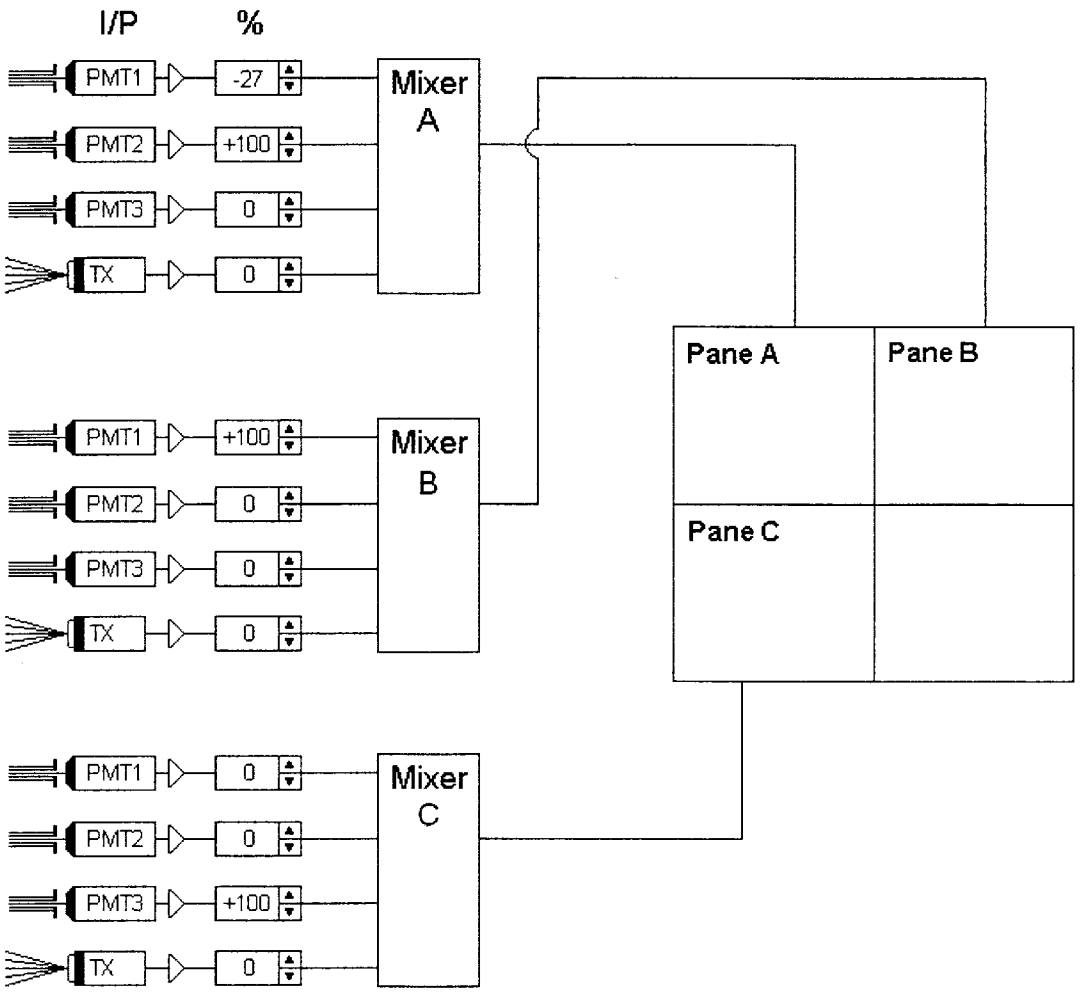


3.3.5 MIXER CONTROL PANEL



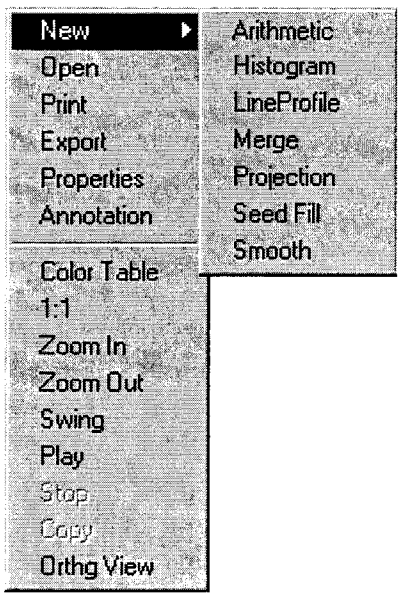
There are three digital Mixers available in the system.

The mixers are particularly useful for **on-line bleed through correction**. The schematic below shows an example of how the mixers would be set to correct for optical bleedthrough of FITC into PMT2. In Mixer A, a fraction of the FITC (green) signal is subtracted from the red signal.


I/P
%
PMT1
-27
PMT2
+100
PMT3
0
TX
0
Mixer A
PMT1
+100
PMT2
0
PMT3
0
TX
0
Mixer B
PMT1
0
PMT2
0
PMT3
+100
TX
0
Mixer C
Pane A
Pane B
Pane C

3.4 Image operators

Image operators (or analysis and processing functions) are accessed via the pop up menu - simply right click on an image and select 'New'.

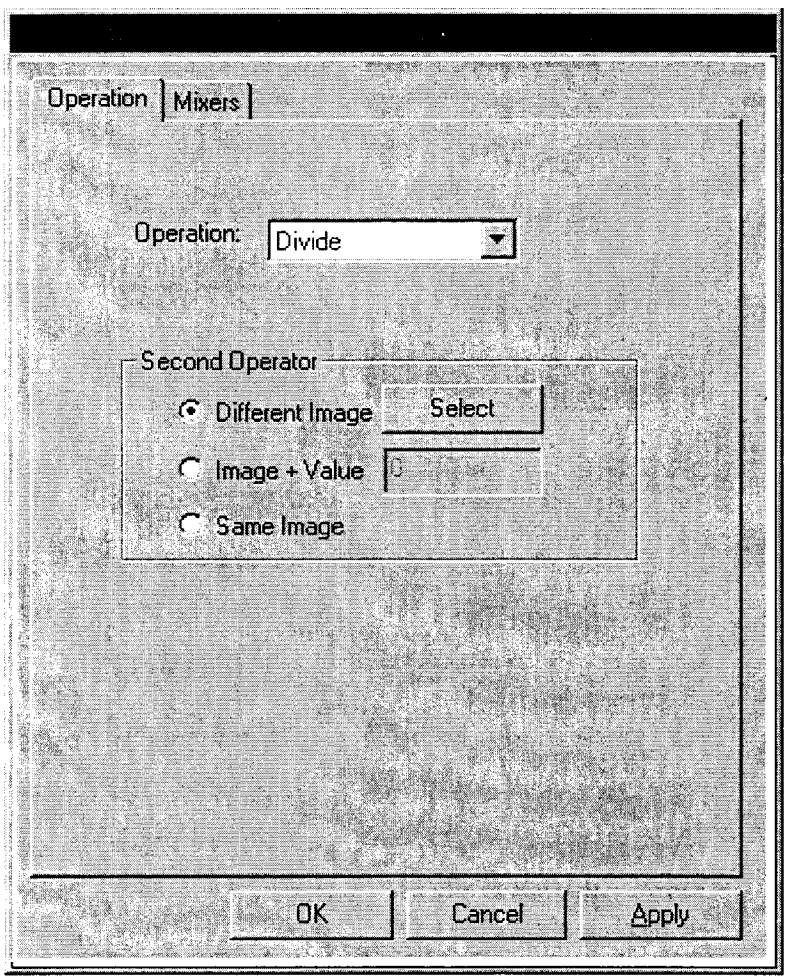


3.4.1 ARITHMETIC

The Arithmetic operator caters for arithmetic operations on images. These include:

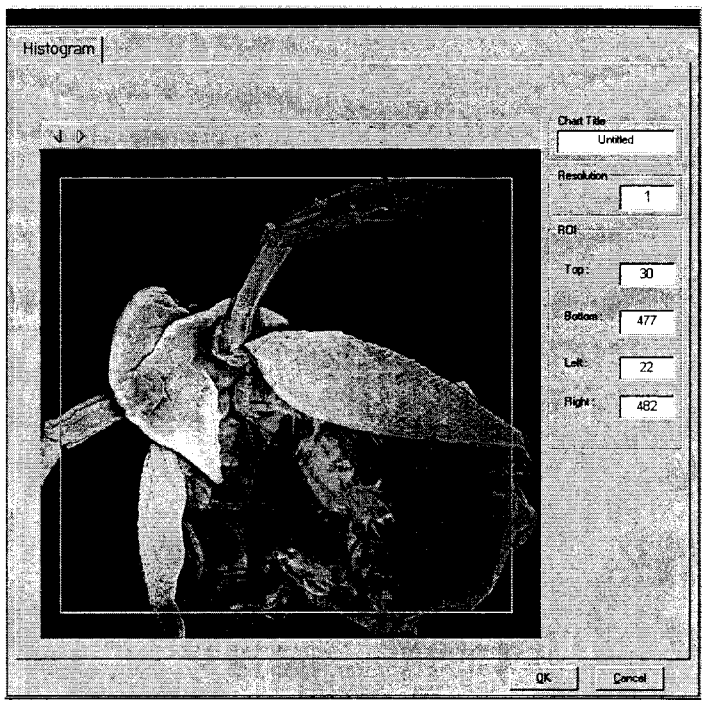
Add
Subtract
Multiply
Divide
Average
And
Or
XOr

Operations can be made between images (e.g. divide one image by another) or upon images by a numerical value (e.g. subtract 20 from all pixel values in an image).

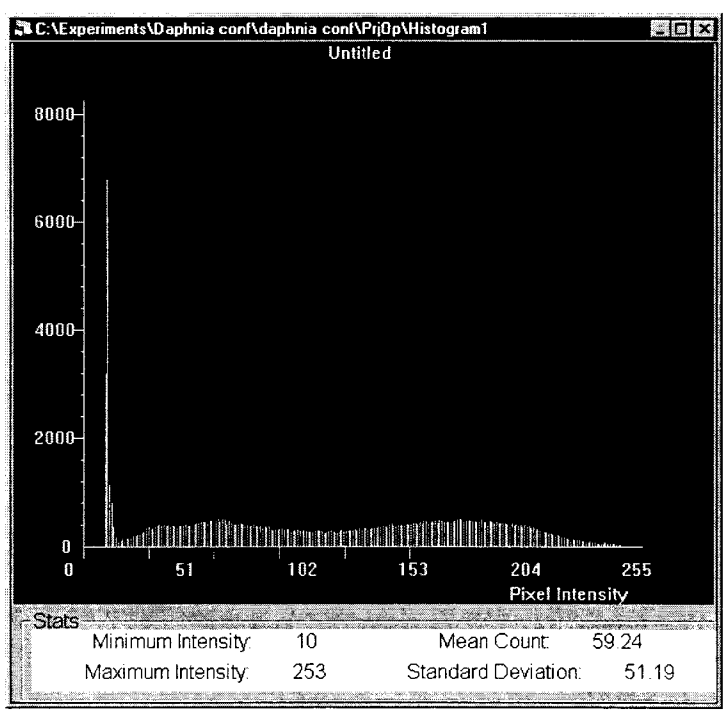


3.4.2 HISTOGRAM

The Histogram operator shows a histogram of all the pixels in the image. The option to show a graph, a table or both is given. The data from the histogram is saved in a file called data.hst in the histogram folder.

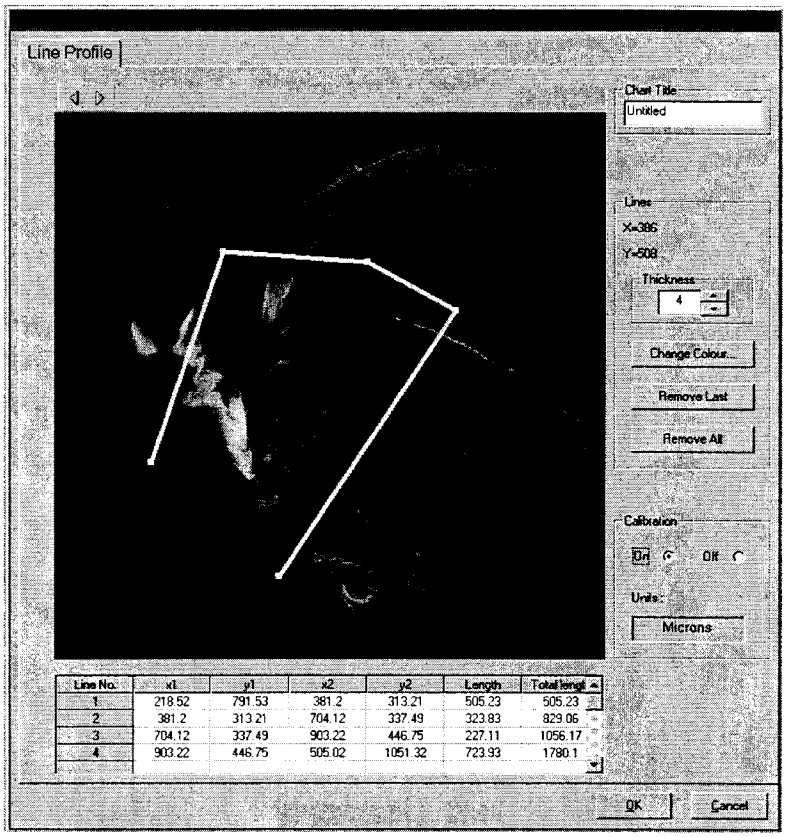


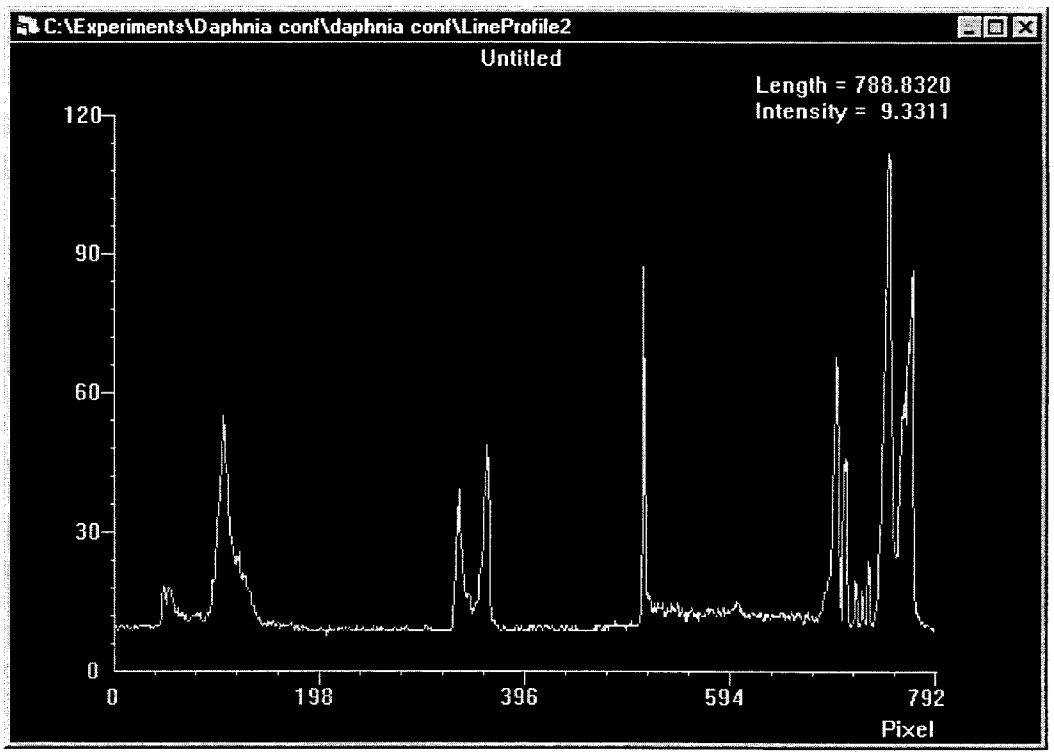
The histogram data will be taken from the whole image by default, but if a rectangle is drawn on the image then the data only from that region will be shown.



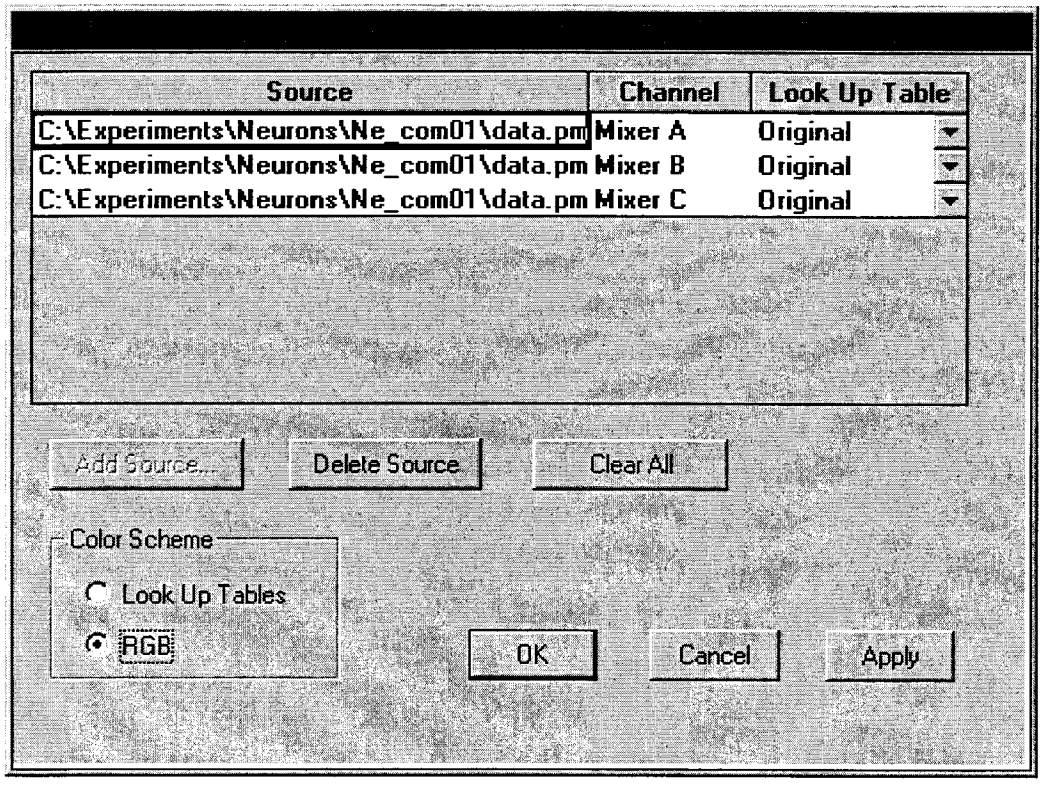
3.4.3 LINE PROFILE

The line profile tool allows measurement of intensity versus XY position. The line thickness can be set to values greater than one to effect smoothing of noisy traces by averaging across the thickness of the line. The data from the line profile measurement is aved in a file called data.lpf in the lineprofile folder.





3.4.4 MERGE

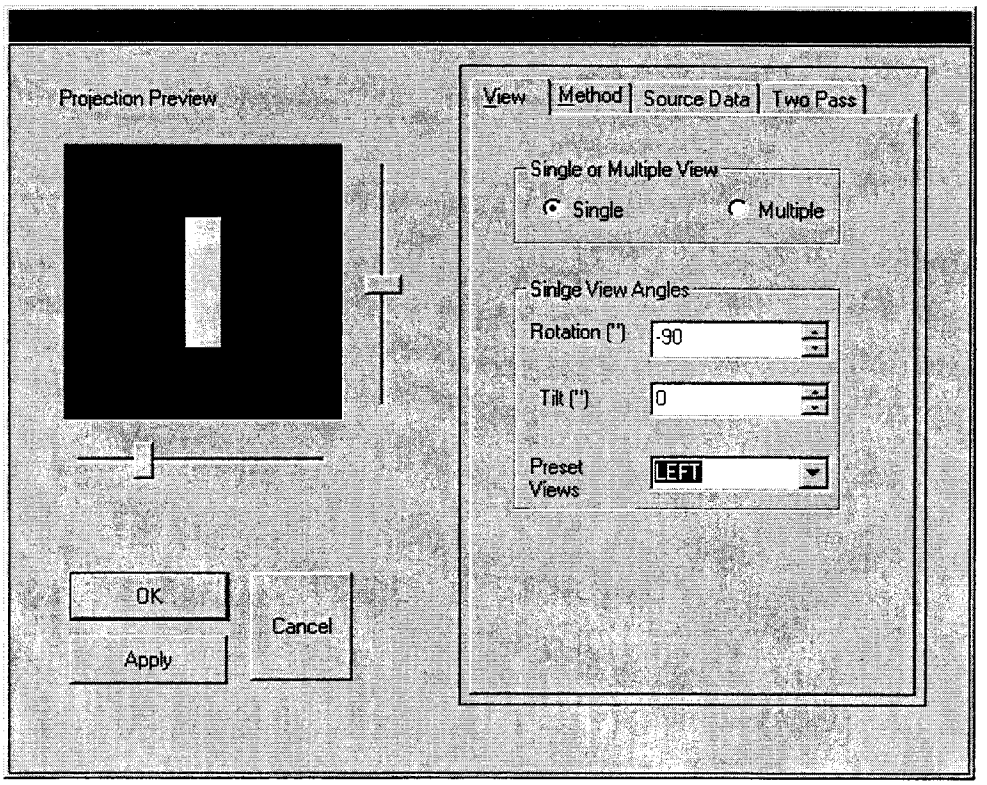


The merge operator can be used to merge upto three images. At present, only images which have been acquired together or have been produced by the Projection operator can be merged.

3.4.5 PROJECTION

The projection operator allows either single or multiple views to be generated using a wide range of projection algorithms.

3.4.5.1 Single view

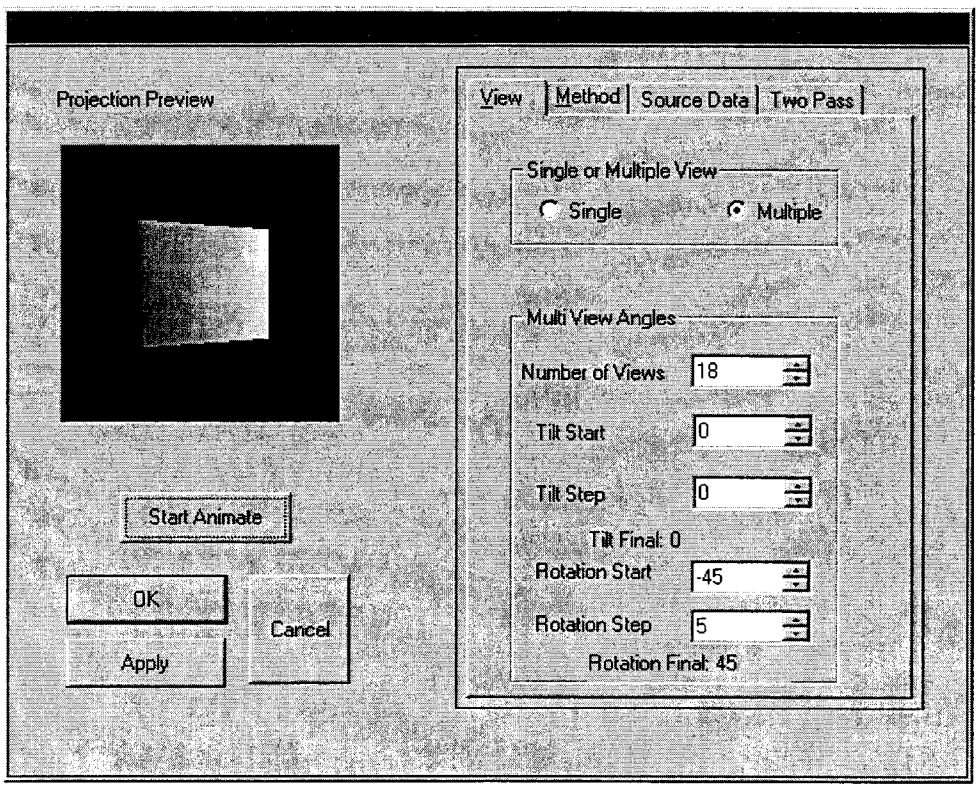


There are six preset views available:

Front
Back
Top
Bottom
Left
Right

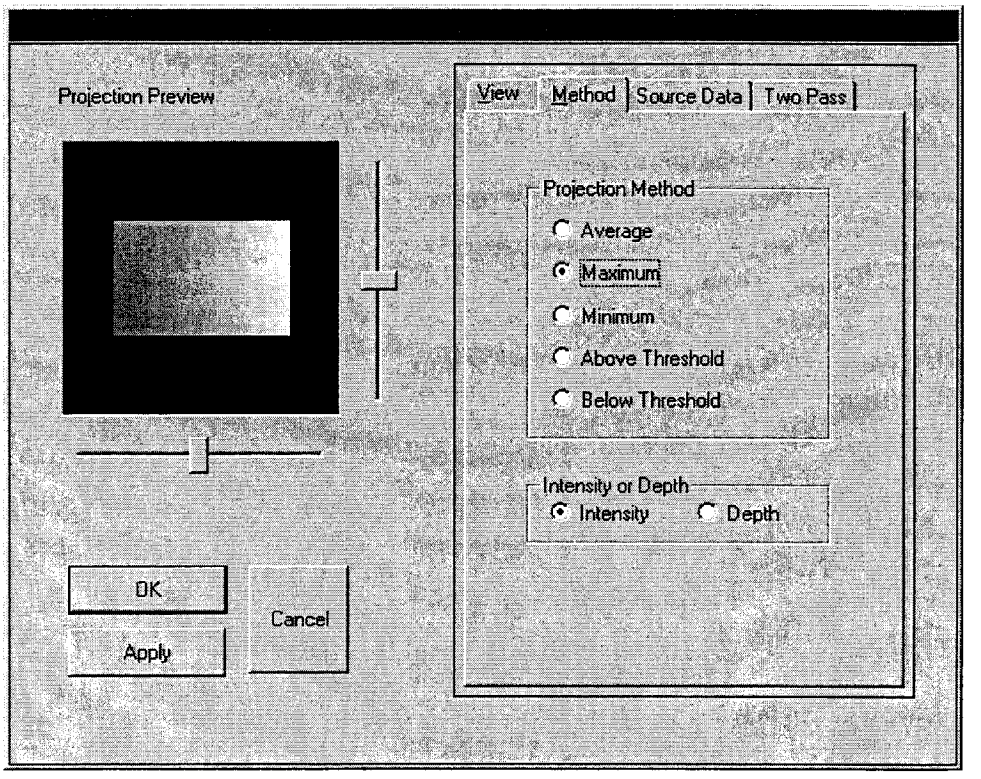
Additionally, a Custom option is given where any angle of tilt or rotation can be selected.

3.4.5.2 Multiple Views



On selecting Multiple Views the extent of the tilt and rotation angles and the steps between the views can set.

3.4.5.3 Projection method (type)



In all of these projection modes imaginary lines (of sight) are traced through the data set and the intensity values of the voxels that lay on each of these lines are used to calculate the resultant single value for that projected line.

There are five projection methods available:

Maximum - Chooses the highest voxel value and ignores the rest. This is very well suited to the majority of Confocal fluorescence data sets, particularly those that have very open structures e.g. Neurones, cytoskeletal labelling or surface labelling of cells.

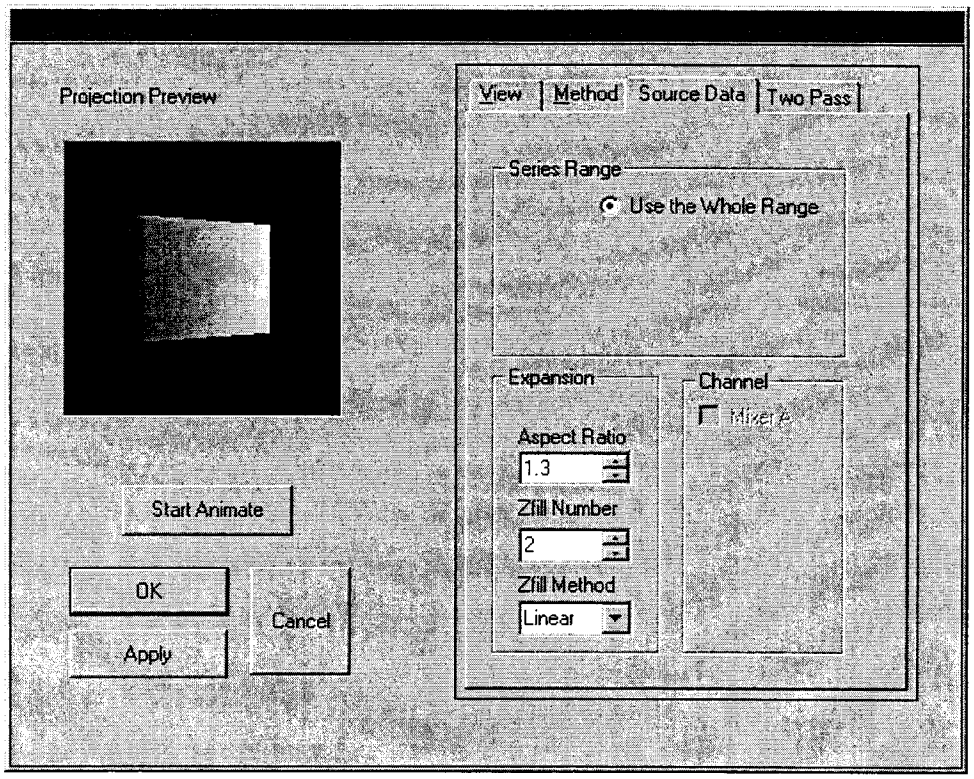
Minimum - Chooses the lowest voxel value and ignores the rest. This can be used to good effect with negative labelling techniques such as imaging living cells in a medium containing FITC-Dextran.

Average - Calculates the mean of all voxel values along the line. Useful where a maximum brightness projection discards important information from the less bright regions of the data set. An average brightness projection will almost always produce a dimmer image than the equivalent Maximum brightness projection. This is because many zero or low brightness values will be included in the averaged values along the line. This can be compensated for by adjusting the Contrast in the image.

Above threshold - Chooses the first voxel value to exceed the selected threshold. This can be useful for imaging an anterior (front) surface in a data set which may not be the brightest feature along that line of sight. A more powerful method of imaging anterior surfaces or features is to use a Two Pass projection as described in the next section.

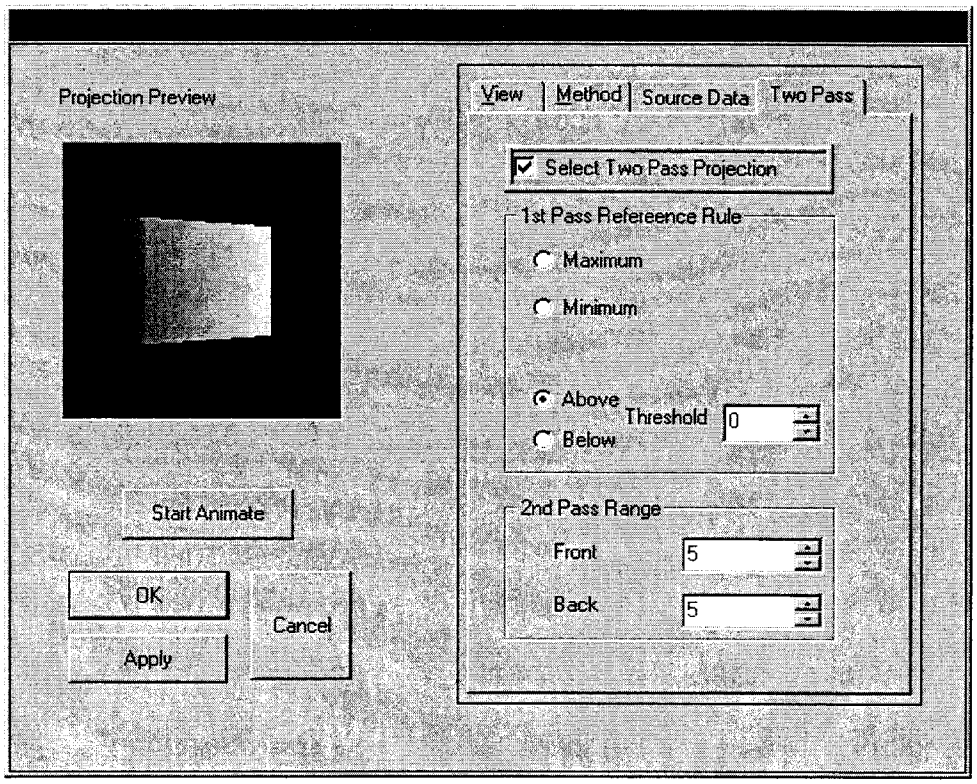
Below threshold - Chooses the first voxel value to fall below the selected threshold. Again, useful for negative staining techniques where one wishes to image the anterior surface of a dark object in a bright surrounding medium.

3.4.5.4 Source data



This page is used to set the z-axis to xy-axes aspect ratio. If the aspect ratio is set to a value greater than 1.0 then a Z-fill value and method are required. The Z-fill value determines the number of additional points which will be added between sections and the method offers the choice of either replicating the preceeding value or using linear interpolation between adjacent values.

3.4.5.5 Two Pass Projections



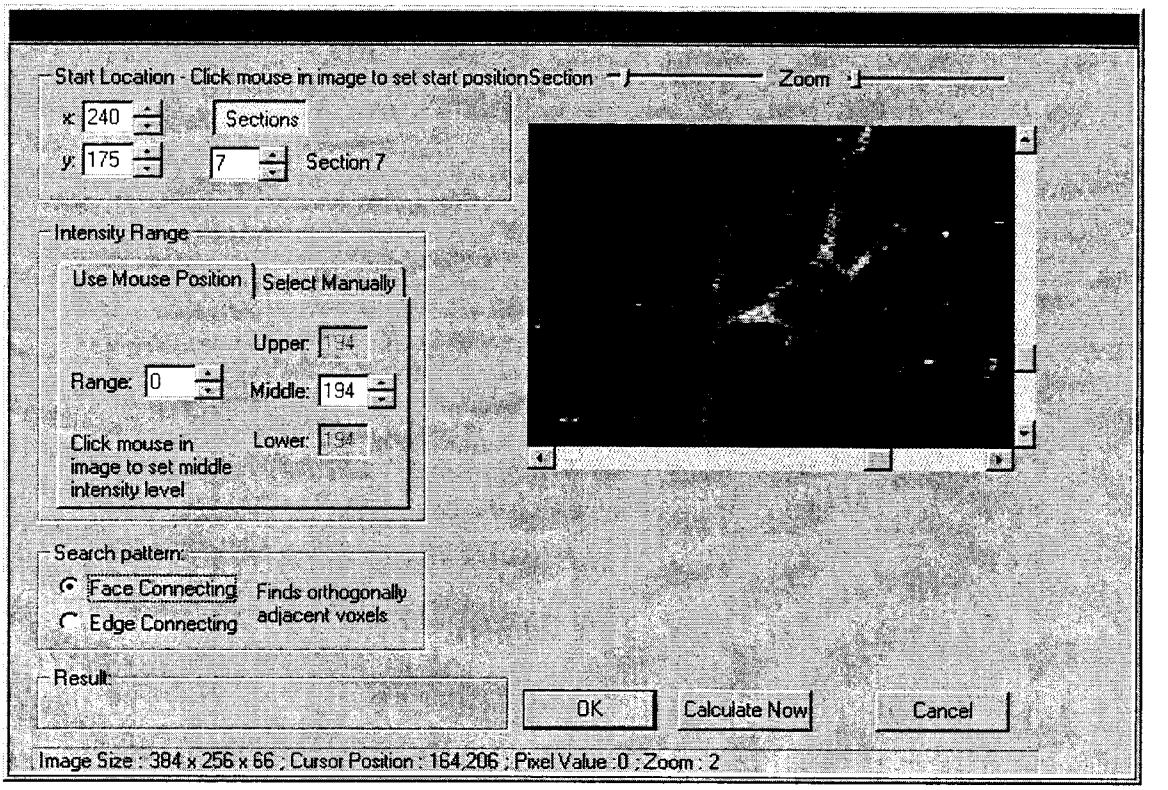
These compound projections can be used to great effect when reconstructing Confocal fluorescence data sets. In the most commonly used single pass projection (maximum brightness) it is clear that two views of a data set from opposite directions (180 degrees opposed) will be exactly the same in everything but orientation. In fact one is a mirror image of the other. If one could 'see' a fluorescence image, confocally, by eye then this would indeed be what we would see. In other words, the sample would be totally transparent. In every day life the vast majority of things that we see and interact with in three dimensions are totally opaque. It is this very 'Opacity' that plays an enormous role in our perception of depth or 3-Dimensionality. In other words, we need a projection algorithm that shows us different views of the sample from front and back.

A two pass projection allows us to achieve this by using the first pass to 'locate' a surface by a selectable criterion (e.g. first voxel above a given threshold) and then to make a local projection (e.g. maximum or average) a limited distance away from that point, towards the viewer or a combination of the two.

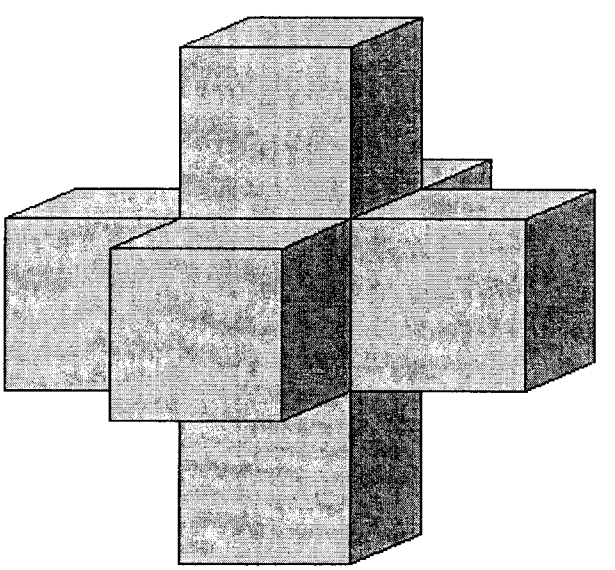
The first pass projection is referred to as the 1st pass reference rule and the second pass as the projection method. The projection methods are exactly the same as those used in single pass projections and are accessed from the same page in the notebook.

The **Single View** projection option is designed to allow the user to quickly ascertain the optimal projection algorithm for a data set prior to generating a set of projections in the batch mode menu.

3.4.6 SEED FILL



Seed Filling uses a simple algorithm to segment pixels (or voxels in 3-D) by the dual criteria of intensity and connectivity. A starting point (the seed voxel) is selected using the mouse pointer and the algorithm checks either all orthoganally adjacent (face connecting) voxels (as shown below), or all surrounding (edge connecting) voxels, to see whether or not they meet the intensity criterion set with the parameters in the dialog box.



If the voxel does meet this criterion then it is selected and written into a new data volume in a new window. Having been selected the same check is made on the new neighbouring voxels, and so on. In this way continuous structures can be separated from complex 3-D volumes.

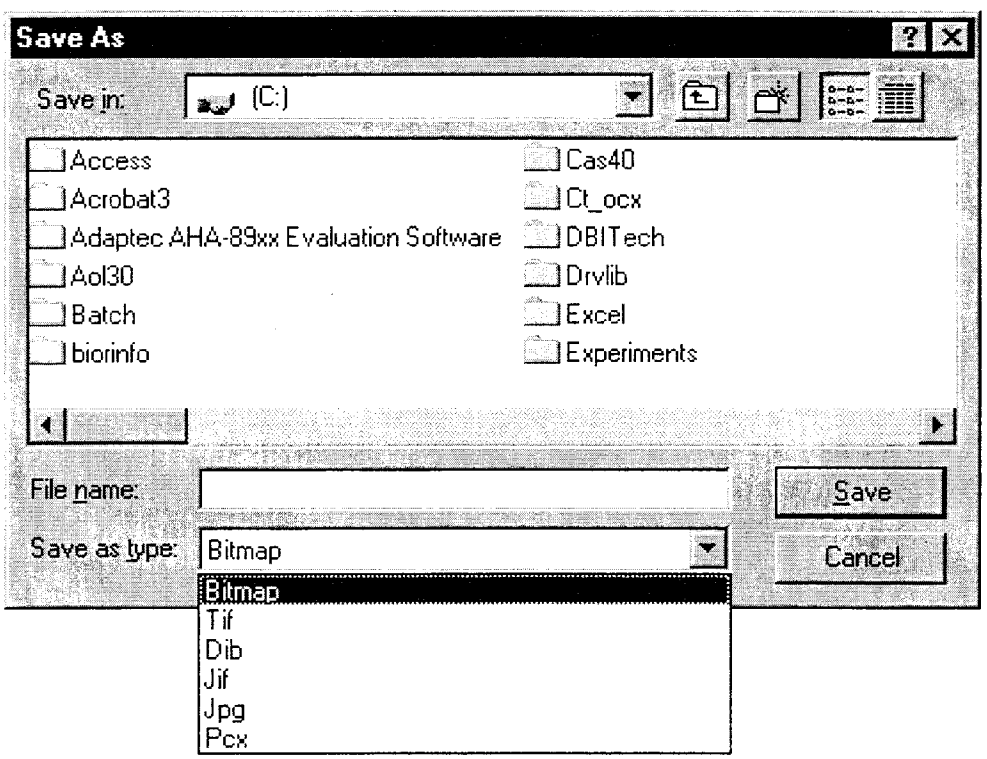
This process works particularly well for neuronal and vascular structures. A particularly useful 'by-product' of this operation is that the segmented structure's volume is directly measured (within the limits set by the contrast and signal/noise in the original image data).

The starting point is selected simply by clicking in the image and this is reported on the left hand side of the dialog box.

The intensity range can be set either by setting the upper and lower valuse manually, or by using the start voxels value and setting a range around that value.

3.4.7 IMPORTING AND EXPORTING FILES

To export a file select Export from the pop-up menu for the image.



The file formats currently supported are shown above.

To import a file right click on an experiment folder in the Experiment Browser

3.5 System calibration

The system will be calibrated at installation and should not require alteration. If you are concerned about system calibration contact your local service representative or email confocal_support@bio-rad.com.